INSIGHT INTO THE GERMINATION MECHANISM OF PSYCHROTROPHIC GROUP II *CLOSTRIDIUM BOTULINUM* (TYPE E) SPORES

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Voorwoord

Zo, dat was het dan. Mijn werk in het labo levensmiddelenmicrobiologie is volbracht. Vier jaar geleden begon ik aan dit heus avontuur, en het is voorbij gevlogen. Ik sluit deze bijzondere periode graag af door even terug te blikken op mijn persoonlijke roller coaster van ups en downs, en door de mensen te bedanken die mij geholpen hebben om dit werk te voltooien. Naast het plezier om bij te dragen aan wetenschappelijk onderzoek, waren het voornamelijk de mensen uit mijn omgeving, die de afgelopen jaren zo mooi hebben gemaakt. Daarom is dit voorwoord aan hun gewijd.

Voor de start van mijn doctoraat, toen ik de eerste keer met Chris kwam praten over mijn onderzoeksonderwerp, waren de plannen nog gericht op *Bacillus* sporen. Tijdens mijn studies vond ik bacteriële sporen helemaal niet interessant, maar Chris overtuigde mij al snel om er toch voor te gaan. Wanneer ik thuis de literatuur doornam, vond ik echter zoveel informatie over *Bacillus* sporenkieming dat ik het onderzoek dat wij voor ogen hadden, al minder uitdagend leek te vinden. Nadat ik mijn twijfelgevoelens met Chris besproken had, werd het plan verschoven naar *Clostridium botulinum*. Een anaeroob organisme dat een dodelijk toxine produceert, waar het labo nog niet eerder mee gewerkt had, en waar wereldwijd nog maar één mutant van was verwezenlijkt… "Oké, super, dat doen we!". Aan enthousiasme en motivatie bij mij meestal geen gebrek. Echter de kunst van het relativeren, kalm blijven, geduldig zijn, die eigenschappen heb ik door de jaren van Chris moeten leren, en om die reden was hij een uitstekende promotor voor mij. Want die skills had ik wel degelijk nodig, er deden zich namelijk al snel heel wat problemen voor. Natuurlijk zijn problemen zeer eigen aan een doctoraat, ze hadden mij hiervoor gewaarschuwd. Ik hoor Joleen nog zeggen "Je moet niet verwachten dat je doctoraat zo'n succes zal zijn als je masterthesis, want er zal héél veel mislukken". Maar bijna een jaar lang wachten op de juiste stammen (omdat het *Clostridium* wereldje niet graag samenwerkt met onbekenden, daarom, geen dankwoordje voor hun!) en anderhalf jaar wachten op een anaeroob werkstation (toch wel écht nodig om *C. botulinum* te kunnen manipuleren, we hebben het ook geprobeerd zonder…), dat was niet de beste start. Laurence, voor jou wel een dikke merci. Door jouw hulp kon ik eindelijk aan de slag met de NCTC 8266 stam. Vanaf toen raasde de roller coaster even de lucht in. Een paar weken voordien stonden Koen en ik in Bocholt nog in het slib van een vijver op zoek naar *C. botulinum* sporen (ja ja, eigen isolatie is ook een plan geweest).

Het was niet eenvoudig om alles van nul op te starten. Welke media, welke protocols, welke materialen, zuurstofproblemen, alles was anders dan wat de rest in het labo deed. Tijdens de eerste maanden dat ik werkte met de toxische stammen ben ik ook verschillende keren bang geweest omdat ik dacht symptomen van verlamming op te merken. Maar het onderzoek ging toch -al was het aan een slakkentempo- vooruit, gelukkig zonder botulisme. Mijn grote doel was een atoxigene mutant te maken, en ik had de eerste jaren nooit verwacht dat ik verder zou geraken dan dat. Met een tevreden gevoel kan ik nu terugblikken op de bekomen resultaten, en ben ik gerustgesteld dat het onderzoek zal worden verdergezet (go go go Aurélie!). Ik ben nu wél een grote fan van sporenonderzoek, "mijn Botje" zal mij zonder twijfel voor altijd bijblijven, net als de mensen uit mijn omgeving.

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De grootste kanjer en degene die mij er al- al- altijd doorsleurde als ik zei dat ik dit onderzoek niet van de grond zou krijgen, is Kristof. Niet enkel de laborant van het labo, maar in mijn ogen het fundament dat alles recht houdt. Zonder hem is ons labo gewoon pure chaos. En voor mij was je nog veel meer. In de eerste plaats was je een ijzersterke hulp om het *C. botulinum* onderzoek opgestart te krijgen (zonder jou zou ik nu nog altijd met anaerobe zakskes aan het werken zijn), en daardoor werden we al snel échte buddies, met een officiële start in Melle. Ik heb mij super goed geamuseerd met u, maar daarnaast stond je ook te allen tijde voor mij klaar, je luisterde naar mij, je begreep mij, je wou altijd helpen. Met uw (zeer aanwezige) humor zorgde je voor groot vermaak op het labo en bij elke LMM uitstap. Ik denk dat ik toch wel de meeste mopkes/plagerijen van u te incasseren kreeg (als Gil dan ook nog in de buurt was, kregen die mopkes een heel specifiek karakter), maar die probeerde ik zoveel mogelijk terug te kaatsen. We hebben vaak hard gelachen en als we samen in het labo waren, was het leute gegarandeerd (ik had de beste plek in 't labo, tegenover u!), maar ook de minder fijne dagen werden beter als jij er was. Ik heb echt zóveel aan u gehad, dat ik het niet kan neerschrijven. Wij zijn vrienden voor altijd. Bedankt voor alles Kristof.

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spreekwoord van mij, kijk eens aan ;)), wij waren een topteam! Het was echt super om alles samen door te maken met u, van begin tot eind, en nu allebei op naar het volgende… Ik wens u het allerbeste toe, met uw volgende job, met Jitka, met de sport. Het waren geweldige jaren, en daar was jij de grootste bijdrage aan.

Een andere topkerel in de hoofdrol, is meneer Alexander Cambré. En die titel mag ervoor want ik vind u echt een grote meneer. Doordat we samen gethesist hadden, -dacht- ik u al te kennen bij de start van ons doctoraat, maar ik had het mis. Ik wist toen nog niet dat ik zoveel aan u zou hebben, en zeker niet dat ik -u- zo graag zou hebben. Ik wil u bedanken voor de vele raad die ik van u heb gekregen in het labo (Cambré weet áltijd raad), en de veeele hulp met de microscoop, maar bovenal ben ik dankbaar dat wij vrienden zijn geworden. Onze meningen of ideeën zijn misschien niet altijd dezelfde, maar ik hou van uw eerlijkheid, en ik kon voor een goed gesprek altijd bij u terecht. Alex, jij hebt een groot hart en bent een super gezellig persoon, waar ik hoop nog mijn leven lang hapje-tapjes mee te mogen doen. Daarom zeg ik nu geen succes voor alles wat nog gaat komen, want ik hoop dat geregeld *in real life* te kunnen doen.

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Voila, allemaal kanjers dus, waardoor alle mislukte experimenten snel goedgemaakt werden en waardoor ik geen één dag met tegenzin ben gaan werken.

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Samenvatting

Clostridium botulinum is een strikt anaerobe sporenvormende bacterie, en produceert het meest dodelijke toxine dat tot op heden gekend is, het botulinum neurotoxine (BoNT). Voedsel dat besmet is met (sporen van) deze pathogeen, kan daarom een groot gevaar vormen voor mens en dier. Bacteriële sporen zijn resistente structuren die zich in een metabolisch inactieve staat bevinden waardoor ze weerstandig zijn tegen allerlei stressfactoren zoals verhoogde temperatuur, droogte, gebrek aan nutriënten, UV, chemicaliën en zuurstof. Wanneer de condities gunstig zijn, kunnen zij uitgroeien tot vegetatieve cellen hetgeen gepaard gaat met toxineproductie. Vermits sporen een complex kiemingsproces moeten doorlopen vooraleer vegetatieve groei mogelijk is, is het in principe mogelijk om uitgroei en toxineproductie te voorkomen wanneer men sporenkieming kan verhinderen. Nochtans is het kiemingsproces van *C. botulinum* sporen nog maar zeer beperkt ontrafeld, voornamelijk omdat onderzoek met deze bacterie wordt bemoeilijkt door risico's met betrekking tot bioveiligheid en bioterrorisme, en omdat strikt anaerobe werkomstandigheden noodzakelijk zijn om de bacterie te manipuleren. In deze doctoraatsthesis werd gefocust op psychrotrofe groep II *C. botulinum* (gIICb), een belangrijke doelwitpathogeen in minimaal behandelde gekoelde kant-en-klare voedingsmiddelen met een lange houdbaarheid.

Om bioveiligheidsredenen werden eerst niet-toxigene mutanten geconstrueerd om het onderzoek naar het kiemingsproces te vergemakkelijken. Hiervoor werd een nieuwe deletiestrategie ontwikkeld waarmee vervolgens een Δ*bontE* mutant van *C. botulinum* NCTC 11219 werd verwezenlijkt. Het betreft de allereerste constructie van een deletiemutant in gIICb, een species groep die genetisch zeer moeilijk te manipuleren is en waarvoor de genetische toolbox nog zeer gelimiteerd is. Daarnaast werd ook een *bontE* insertiemutant gecreeërd in dezelfde parentale stam, via het ClosTron systeem. De groei- en sporulatiekarakteristieken alsook de hitteresistentie van de sporen van beide mutanten bleken grotendeels identiek te zijn aan de wildtype stam. Echter, ook enkele fenotypische verschillen werden waargenomen, en deze werden toegeschreven aan bijkomstige mutaties die geïdentificeerd werden via *whole genome* sequentieanalyse. Daarnaast kon uitgesloten worden dat de fenotypische verschillen gerelateerd zijn met de uitschakeling van BoNT-productie, doordat dezelfde atoxigene mutanten (via deletie en insertie) werden aangemaakt in een andere *C. botulinum* stam (NCTC 8266). Deze mutanten vertoonden geen verschil met de parentale stam, behalve een groeidefect bij verlaagde temperatuur voor de ClosTron mutant. De deletiemutanten van beide stammen bieden interessante perspectieven als veilige surrogaatorganismen voor gIICb in *challenge testing* van voedingsmiddelen.

In een tweede onderdeel van dit doctoraatsonderzoek, werd de sporenkieming in meer detail onderzocht. Hiervoor werd eerst het kiemingsinducered effect getest van het nutriëntmengsel L-alanine/L-lactaat/$NaHCO_3$, hoge-druk-behandeling, calciumdipicolinaat (Ca^{2+}-DPA) en dodecylamine. Op basis van het verlies van hitteresistentie en de vrijzetting van DPA werd geconcludeerd dat enkel de nutriënten en het surfactant dodecylamine sporenkieming induceerden. Dodecylamine-gekiemde sporen behielden echter hun refractiliteit in fasecontrastmicroscopie, hetgeen betekent dat ze geen water hebben opgenomen. Deze sporen waren ook enkel hittegevoelig in aanwezigheid van dodecylamine, en niet meer wanneer dodecylamine eerst werd weggewassen. Dit wijst er op dat dodecylamine geen echte kieming induceert.

Vervolgens werd het kiemingsinhiberend effect van vier natuurlijke antimicrobiële componenten (carvacrol, kaneelaldehyde, essentiële olie van wortelzaad en hop β-zuren) onderzocht. Hoewel natuurlijke componenten voornamelijk bestudeerd zijn voor hun groei-inhiberend effect, kan kiemingsinhibitie ook een bijdrage leveren tot de controle van sporenvormende bacteriën in voedingsmiddelen. De resultaten toonden dat de minimale concentratie nodig voor een significante vermindering van nutriëntkieming, vele malen lager ligt dan de minimale inhibitorische concentratie (MIC) op vegetatieve cellen voor carvacrol, kaneelaldehyde en essentiële olie van wortelzaad. Omdat relatief lage smaak- en geur-grenswaarden de applicatie van deze componenten in voeding vaak in de weg staan, zouden lagere concentraties dus mogelijk effectief kunnen zijn om gIICb sporenkieming te controleren.

In een meer mechanistisch gericht gedeelte, werden via deletieanalyse specifieke genen onderzocht die voorspeld worden een rol te spelen in het kiemingsproces van gIICb. De aanwezigheid van slechts één Ger-type germinant receptor, GerX3b, in gIICb stammen is atypisch omdat andere sporenvormers doorgaans meerdere dergelijke receptoren hebben. Het is bovendien intrigerend, omdat onduidelijk is hoe één receptor kan reageren met de vele verschillende nutriënten die kieming van gIICb sporen kunnen induceren. De *gerBAC* gencluster, die codeert voor de drie subeenheden van de GerX3b receptor, kon succesvol worden gedeleteerd. Kiemingsexperimenten met deze deletiemutant toonden aan dat de GerX3b receptor geen rol speelt in kieming geïnduceerd door verschillende nutriëntmengsels. Verder werd getracht twee voorspelde cortex hydrolasen, SleB and SleC, uit te schakelen met dezelfde deletiestrategie. Enkel voor SleB was dit succesvol. Sporen zonder SleB bleken nog steeds in staat om te kiemen, waaruit geconcludeerd kan worden dat dit enzym niet essentieel is om de cortex te hydrolyseren, zoals eerder voor SleB van *C. difficile* werd gerapporteerd.

Een beperkte rol in sporenkieming kan echter niet worden uitgesloten omdat de kiemingsexperimenten een grote variabiliteit vertoonden.

Summary

Clostridium botulinum is a strictly anaerobic spore-forming bacterium, and produces the most deadly toxin known, the botulinum neurotoxin (BoNT). Therefore, food contaminated with (spores of) this pathogen constitutes an important threat to humans and animals. Bacterial spores are highly resistant dormant structures that are resilient against many hostile conditions like heat, dryness, lack of nutrients, UV, chemicals and oxygen. When conditions become favorable, they can grow out and resume vegetative growth, which is accompanied with toxin production. Since spores first have to complete a complex cascade of events designated as germination, before they can grow out, outgrowth and toxin production can in principle be prevented by inhibition of germination. However, the germination mechanism of *C. botulinum* spores has only been unraveled in very little detail, mainly due to biosafety and bioterrorism restrictions and because a strict anaerobic environment is required to grow the pathogen. In this PhD thesis, we focused on psychrotrophic group II *C. botulinum* (gIICb), the major pathogen of concern in minimally processed chilled ready-to-eat foods with extended shelf life.

Due to biosafety reasons, nontoxigenic mutants were first constructed to facilitate the research on the germination mechanism. To this end, a novel deletion strategy was developed and used to create a Δ*bontE* mutant in *C. botulinum* NCTC 11219. This was the first report of a deletion mutant in gIICb, a species group that is known to be less genetically tractable than other clostridia, and for which the genetic toolbox is still very limited. In addition, a *bontE* insertional knockout mutant was created by the ClosTron system. While most characteristics of the mutants showed to be identical to the parental strain, also some differences were noted. Presumably, these changes are due to some spontaneous mutations that were identified by whole genome sequence analysis. It could be excluded that the changed properties are associated with BoNT inactivation, since the same atoxigenic mutants were constructed (by insertion and deletion) in a different parental strain (NCTC 8266) and these did not show any altered properties, except for a growth defect at lowered temperatures for the ClosTron mutant. The deletion mutants of these two strains offer safe surrogate organisms for gIICb in challenge testing of foods.

In a second part of this PhD research, the spore germination process was investigated. First, the ability to induce germination of the nutrient mixture L-alanine/L-lactate/$NaHCO_3$, high pressure treatment, calcium dipicolinic acid (Ca^{2+}-DPA) and dodecylamine was assessed. Based on the loss of heat resistance and the release of DPA, it was concluded that only the

nutrient mixture and the surfactant dodecylamine could induce germination. However, dodecylamine germinated spores did not loose their refractility in phase-contrast microscopy, indicating that they had not rehydrated. In addition, these spores only became heat sensitive when dodecylamine was present, whereas they remained resistant when dodecylamine was washed away before the heat treatment. This suggests that dodecylamine does not induce genuine germination.

Furthermore, four natural antimicrobial compounds (carvacrol, cinnamaldehyde, carrot seed essential oil and hop β-acids) were tested for their inhibitory action on germination. Although these compounds have mainly been studied for their growth inhibiting action, the inhibition of spore germination could also contribute to the control of spore-formers in foods. The results demonstrated that the minimal concentration of carvacrol, cinnamaldehyde and carrot seed essential oil that could significantly reduce nutrient germination, was many times lower than the minimal inhibitory concentration (MIC) on vegetative growth. This could be of interest because the low sensory- and odor thresholds often interfere with the application of these compounds in foods.

In a more mechanistically oriented part, specific genes that are predicted to encode proteins involved in germination of gIICb, were investigated via deletion analysis. The presence of only one Ger-type germinant receptor, GerX3b, in these strains is unusual because other spore-formers generally contain more receptors. Moreover it is intriguing, because it is unclear how a single receptor can respond to the many different nutrients that can induce germination in gIICb. The *gerBAC* gene cluster, encoding the three subunits of the GerX3b receptor, was successfully deleted. Germination experiments with this mutant showed that the GerX3b receptor is not involved in germination induced by different nutrient mixtures. Furthermore, two predicted cortex hydrolases, SleB and SleC, were investigated via the same knockout strategy, but a deletion mutant could only be successfully created for SleB. Because spores lacking SleB could still complete cortex hydrolysis, it was concluded that SleB is not an essential cortex hydrolase in gIICb, similar to what has been reported for SleB of *C. difficile*. Nevertheless, an auxiliary role in other germination events can not be excluded since the germination assays showed a large variability.

List of abbreviations

5-FOA	5-fluoroorotic acid
Agr	accessory gene regulator
BoNT	botulinum neurotoxin
Cm	chloramphenicol
CLE	cortex lytic enzyme
c-di-GMP	cyclic dimeric guanosine monophosphate
CDC	U.S. Center for Disease Control and Prevention
Cy	cycloserine
D-value	decimal reduction time
DPA	dipicolinic acid
E-cad	epithelial cadherin
Em	erythromycin
EO	essential oil
FDA	U.S. Food and Drug Administration
gDNA	genomic DNA
gerMix	germinant mixture: L-alanine/L-lactate/$NaHCO_3$
GR	germinant receptor
gICb	group I *C. botulinum*
gIICb	group II *C. botulinum*
HA	hemagglutinin
HP	high hydrostatic pressure
MIC	minimal inhibitory concentration
REPFEDs	refrigerated processed foods of extended durability
MLD50	mouse lethal dose
NTNH	non-toxic-non-hemagglutinin
PASTA	penicillin and ser/thr kinase associated
PG	peptidoglycan
PHAST	phage search tool
PBS	phosphate buffered saline
PTC	progenitor toxin complex
RCM	reinforced clostridial medium
RAM	retrotransposition-activated marker

SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Sp	spectinomycin
TCS	two-component regulatory system
Tm	thiamphenicol
TPGY	trypticase peptone glucose yeast extract
WGS	whole genome sequence

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Chapter 1

Botulism and *Clostridium botulinum*

1.1 Historical overview, from sausage poisoning to foodborne botulism

In 1895, an outbreak of what was then called "sausage poisoning" occurred in the small Belgian village Ellezelles [1,2]. Thirteen persons became poisoned after eating a meal with pickled and smoked ham, and three of them died. After examination of the food and the victims, Emile Van Ermengem, a microbiologist at the University of Ghent, isolated the strictly anaerobic microorganism causing the illness and named it *Bacillus botulinus*. Sausage poisoning, also named 'botulism' after the Latin word *botulus* (sausage), was a known disease at that time, described in detail by Kerner (1786-1862). This German physician made several important observations about the poison: it was commonly found in spoiled sausages where it develops under anaerobic conditions, it interrupts motor neuron signal transmission in the peripheral and autonomic nervous systems, and is lethal in small doses. He also accurately described the symptoms, which include vomiting, intestinal spasms, mydriasis, ptosis, dysphagia and respiratory failure. Interestingly, Kerner speculated about the use of small doses of the poison as a therapeutic treatment for hyperexcitability of the nervous system, an application that has been developed about a century later.

After Van Ermengem's discovery, the botulinum toxin producing pathogen could be linked to more foodborne intoxications, and it was discovered that different strains could produce serologically distinct variants of the toxin (further discussed in 1.2).

1.2 The botulinum neurotoxin (BoNT)

The botulinum neurotoxin (BoNT) is produced by *Clostridium* (*C.*) *botulinum*, and also by some strains of *C. baratii*, *C. butyricum* and *C. argentinense* as a single inactive polypeptide chain of 150 kDa. The protein is activated by a specific proteolytic cleavage within a surface-exposed loop subtended by a disulfide bridge. The active neurotoxin thus consists of a heavy chain (100 kDa) and a light chain (50 kDa) which remain associated via protein-protein interactions and covalently bound by the interchain S-S bond. Until exposed to reducing conditions, such as in the neuronal cytosol, the two chains remain linked [3,4].

The heavy chain comprises a receptor-binding and a translocation domain, by which the toxin binds to high-affinity receptors on peripheral nerve endings and translocates by endocytosis to its cytosolic target, respectively (Fig. 1.1). Further, it is believed that the heavy chain subsequently mediates translocation of the light chain from the endosomes into the cytosol, where it acts as a zinc endopeptidase that cleaves SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor). A thioredoxin reductase-thioredoxin system in the cytosol is responsible for cleavage of the BoNT disulfide bond, enabling toxicity of the

light chain [5]. The SNAREs form a complex which mediates the fusion of neurotransmitter-containing vesicles to the neuron's presynaptic membrane (Fig. 1.1 A). When the BoNT light chain cleaves these SNAREs, no neurotransmitter can be released, resulting in muscle paralysis (Fig. 1.1 B) [3,6]. To date, seven serotypically distinct BoNTs (type A-G) which cleave different SNARE proteins have been identified: BoNT/A and E cleave synaptosome-associated protein (SNAP-25), BoNT/B, D, F and G cleave vesicle-associated membrane protein (VAMP), also known as synaptobrevin. BoNT/C is unique because it is able to cleave both SNAP-25 and syntaxin, a target membrane protein. The amino acid sequences of the seven toxin serotypes differ by 35 to 70 % [7,8].

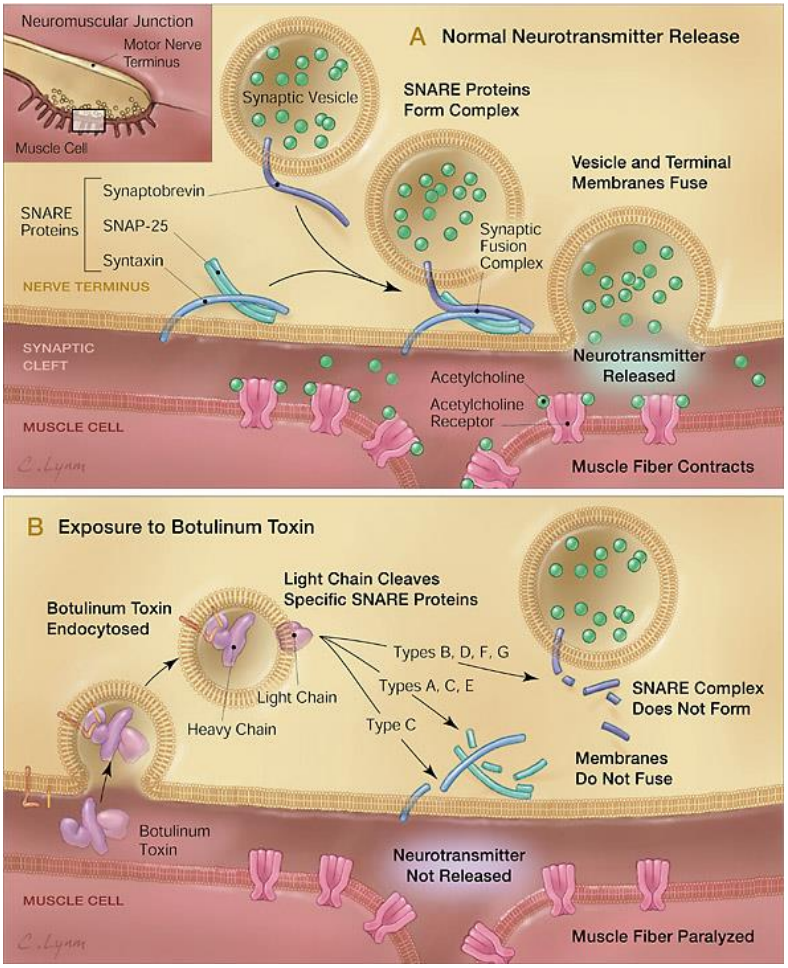

Fig. 1.1: The mode of action of BoNT. A) In case of normal neurotransmitter release, SNAREs enable vesicles containing acetylcholine to dock to the neuron's presynaptic membrane. B) When BoNT reaches the high-affinity receptors on the peripheral nerve endings, the heavy chain ensures translocation of the toxin by endocytosis, whereafter translocation of the light chain into the cytosol occurs. In the neuronal cytosol, the light chain acts as a zinc endopeptidase that cleaves the SNAREs. Consequently, acetylcholine-containing vesicles can no longer dock to the membrane, resulting in muscle paralysis. Source: [8]

The seven serotypes are further subdivided in forty subtypes in which amino acid differences may be minor (1–7 % difference among type B) or major (3–36 % among type F) [9]. BoNT/C and D are closely related, as are types B and G, and E and F. Some *C. botulinum* strains produce

more than one serotype, i.e. type A together with type b, A with f, B with a, or B with f, where capitalized letters refer to the predominant toxin type. Several *C. botulinum* strains producing type A toxin have been found to also encode a silent *bont/B* gene that does not express active toxin due to mutation or truncation. They are denoted A(B) [10]. In 2013, a *C. botulinum* strain with three *bont* gene clusters (subtypes A2, F4 and F5) was reported [11], but this remains exceptional. Also mosaic toxins have been described, for example BoNT/DC which comprises a light chain of serotype D and a heavy chain of serotype C, or BoNT/CD with the converse configuration. Recently a novel BoNT serotype was reported, designated BoNT/H, and this was the first new toxin type since the discovery of type G about forty years ago. However, a genetic comparison with known *bont* sequences suggested that the gene is a hybrid containing regions of BoNT/A1 and BoNT/F5, and the toxin was correspondingly renamed mosaic BoNT/FA [12].

In all botulinum producing clostridia, the *bont* gene is located in a 12-15 kb gene cluster where it is preceded by a set of accessory genes. In some strains the cluster is located on a plasmid or on a prophage. Two primary cluster types are identified: a hemagglutinin (*ha*) cluster and an *orfX* cluster (Fig. 1.2). The type B, C, D and G *bont* genes are organized in a *ha* cluster, whereas the type E and F genes are in an *orfX* cluster. Only *bont/A* can occur in either type of arrangement [13–15]. The *ha* cluster comprises two transcriptional units. One unit encoding a transcriptional activator (*botR*), non-toxic-non-hemagglutinin (*ntnh*) and the *bont* neurotoxin gene. The second unit, transcribed in the opposite direction, encodes three *ha* genes *ha33*, *ha*17 and *ha*70, named according to the molecular weight in kDa of their protein products. BotR drives transcription of both the *ntnh-bont* and *ha* operons, but cannot promote transcription of the individual genes [16]. The *orfX* cluster has a somewhat similar organization: one transcriptional unit encodes the gene *p47* of unknown function, *ntnh* and *bont*, and the second unit consists of three open reading frames of unknown function, *orfX1*, *orfX2* and *orfX3*, but lacks the *ha* genes. Interestingly, the *orfX* cluster of all *bont/E* subtypes, as well as of *bont/F6* and *bont/F7* lacks the *botR* gene and, based on the available genome sequences, *botR* is absent from their whole genomes. It is not known how BoNT expression is regulated in these types nor if *p47* might play a role in regulation. The intergenic regions of some *orfX* clusters also contain insertion elements.

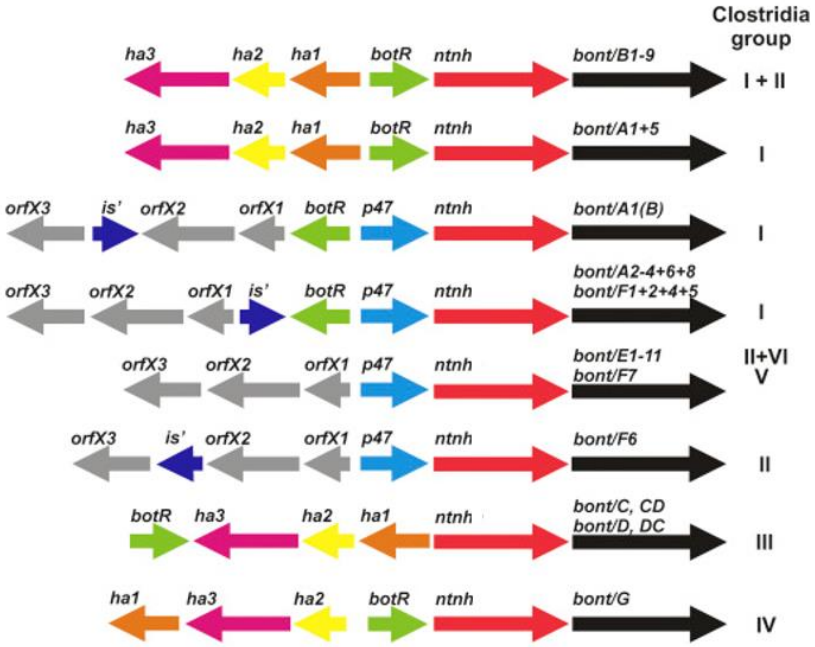

Fig. 1.2: Schematic overview of different BoNT gene clusters. Two main types have been described: a hemagglutinin (*ha*) cluster is present in type A1, A5, B, C, D and G producing strains, whereas an *orfX* cluster is present in type A1-4, E and F producing strains. Varying gene arrangements per cluster are shown and the presence of insertion elements (is') is indicated. *ha33*, *ha17* and *ha70* are shown as *ha1*, *ha2* and *ha3*. *bont/A* is encoded in either type of cluster, due to a horizontal recombination of *ntnh* + *bont/A* within the *bont/B* cluster. Groups I-IV represent *C. botulinum*, group V represents *C. baratii* and group VI represents *C. butyricum*. Adapted from [17]

1.3 Regulation of *bont* expression

It has been observed that *botR/A* (i.e. the *botR* gene in BoNT/A producing strains) is expressed together with *bont/A*, *ntnh* and *ha* predominately during the transition from the exponential to the stationary growth phase [18]. *botR/A* acts as a specific alternative sigma factor that activates the two operon promotors in the *bont/A* gene cluster upon association with RNA polymerase core enzyme [16]. BotR/A seems to be related to a new subgroup of the sigma-70 family, that also includes TetR, TcdR and UviA, which respectively regulate production of tetanus neurotoxin in *C. tetani*, toxins A and B in *C. difficile* and the bacteriocin BCN5 in *C. perfringens* [16]. Moreover, Marvaud et al. (1998) showed that BotR/A and BotR/C can functionally regulate *in vivo* expression of the tetanus neurotoxin gene *tetX* in *C. tetani* [19]. Therefore it has been proposed that this new group of alternative sigma factors mediate toxin and bacteriocin production in several pathogenic clostridia via a common mechanism. In addition, three two-component regulatory systems (TCSs) positively control the expression of *bont/A*, *ntnh* and the *ha* genes, and one additional TCS negatively regulates expression of these genes in the model strain *C. botulinum* Hall A [20,21]. Furthermore, BoNT production is regulated by quorum sensing. Quorum sensing accessory gene regulator (Agr) systems regulate bacterial cell density by measuring the concentration of small autoinducers. In ten *C.*

botulinum (group I) genomes two *agr* loci were identified, *agr-1* and *agr-2*. Both were maximally expressed during the late exponential phase. While *agr-1* seems to control sporulation, *agr-2* is proposed to regulate BoNT production [22]. In addition, there may also be a link between sporulation and toxin production, since insertional inactivation of Spo0A, which is the master regulator in sporulation (further discussed in 2.1.1), resulted in reduced synthesis of BoNT in three type E strains besides loss of sporulation [23]. Furthermore, Zhang et al. (2014) demonstrated that also CodY positively regulates the *ntnh-bont* transcript and BoNT production in *C. botulinum* Hall A [24]. CodY is conserved amongst the *Firmicutes* and controls more than hundred genes in *B. subtilis* by sensing levels of GTP and branched-chain amino acids [25–27], and it determines the cell to adapt during transition of exponential growth to stationary phase. In addition, CodY controls virulence gene expression in *C. difficile*, *C. perfringens*, *B. cereus*, *Listeria monocytogenes* etc. [28–31]. CodY-dependent regulation of BoNT production was GTP-dependent, suggesting that it is associated with nutritional status [24].

1.4 The BoNT accessory proteins

BoNT associates with NTNH in a 1:1 molecular stoichiometry to form a noncovalent BoNT/NTNH complex. Furthermore, in the strains that contain the *ha*-type neurotoxin cluster, three HA-70, three HA-17, and six HA-33 molecules associate with this complex. Together they form the progenitor toxin complex (PTC), which is the largest bacterial toxin complex known.

After oral ingestion, NTNH protects BoNT against acid and proteolytic degradation in the gastrointestinal tract of animals and humans. *In vitro* studies showed that free BoNT is much more easily detoxified by pepsin, pancreatin, and by gastric and intestinal juices [32,33]. As a result, without formation of the complex, the oral lethal dose of free BoNT/A is about ten- to hundredfold higher [32,34,35]. Protection is accomplished because of NTNH's similar crystal structure to BoNT, which enables the formation of a tight NTNH/BoNT complex in which a large surface area is buried [36]. Acidic conditions are essential to achieve this mutual fit, because acidity induces a specific conformation in NTNH. Moreover, specific pH-sensing residues on both proteins allow electrostatic interactions that strengthen the complex. After passage of the stomach, the toxin has to transfer through the intestinal wall to reach circulation. It has been shown that the HAs facilitate transcytosis of BoNT across this barrier by interacting with the epithelial cells [37,38]. Without the HA complex, NTNH/BoNT is also capable of transcytosis, but less efficiently, and thus resulting in lower oral toxicity [32]. The HA complex is believed to anchor the PTC to the intestinal wall by interacting with highly glycosylated proteins on the epithelial cell surface, using multiple carbohydrate-binding sites. This model

is supported by the observation from mouse oral toxicity assays showing that carbohydrate receptor mimics extended the survival of the mice after intake of a lethal BoNT/A dose [39]. Additionally, a PTC that was mutated in carbohydrate binding showed drastically reduced oral toxicity [40]. Furthermore, the HA-glycan interaction shows some serotype specificity, which may contribute to the observed different oral toxicity and host susceptibility among different BoNT serotypes [41,42]. In mice, the receptor for PCT/A was recently shown to be glycoprotein 2 of the microfold (M) cells [38].

Subsequent to adhesion to the intestinal cells, the complex is transcytosed through the epithelial layer. Once the PTC is transported to the basolateral side, the HA complex interacts with epithelial cadherin (E-cad) on the basolateral side, resulting in a disrupted barrier which enables paracellular influx of PTC [43]. The interaction between the HA proteins and E-cad is serotype- and species-specific: the HAs of BoNT/A and B bind efficiently to human, bovine, or mouse E-cad, but not to chicken E-cad. Conversely, the HA of BoNT/C does not recognize human E-cad. This observation correlates well with the finding that human foodborne botulism is rarely associated with BoNT/C, but mostly with types A, B, E and F, while avian botulism is linked to types A and C, and ruminant botulism to types C and D. However, host specificity is probably not only determined by the HA-E-cad interaction, but the additional mechanisms are poorly understood.

After transcytosis, the PTC reaches systemic circulation, where BoNT and NTNH dissociate from the complex because the transition from acidic to neutral or alkaline pH causes deprotonation of specific sensor residues in BoNT. It is currently investigated whether these unique properties of these accessory proteins can be exploited to shield certain therapeutic proteins and promote their active uptake during gastrointestinal passage. [44]

Interestingly, the HA genes are absent in BoNT/E, BoNT/F and BoNT/A2 strains. These strains contain the *orfX* cluster, but the expression and function of the OrfX proteins are unknown. It has been suggested that their function is similar to the HA proteins because of their analogous location and orientation in the genome. However, further studies are definitely required to elucidate the intestinal absorption mechanism of these BoNTs.

1.5 Botulism

The BoNT producing clostridia are widespread in soil and sediments of rivers and lakes. Moreover, clostridia are sporeforming bacteria, of which the spores can persist for decades. Because of the strictly anaerobic metabolism of clostridia, their spores can only grow out to vegetative cells and produce toxin in the absence of oxygen. Decomposition of plants, algae, invertebrates, and animals creates such anoxic environments. From these niches, outbreaks of animal botulism can spread rapidly, leading to the intoxication of thousands of animals in only a few days [10]. What causes human botulism and how it is treated, is discussed below.

1.5.1 Different forms of human botulism

Dormant spores from the environment are unlikely to cause human botulism orally because healthy adults and children over one year of age have a mature digestive system that prevents the survival of germinated *C. botulinum* spores, whereas the dormant spores will pass through the digestive system.

Foodborne botulism occurs when food is contaminated with spores that are able to grow out and produce toxin in the food, and if the toxin is not inactivated by heat treatment before consumption. Woodburn et al. (1979) demonstrated that 10^5 MLD_{50} (mouse lethal dose causing 50 % mortality) of BoNT type A, B, E and F was reduced to undetectable levels (with the mouse bioassay) by heating foods at 79 °C for 20 min, or at 85 °C for 5 min, and suggested that these heat treatments could be used as a guideline for safe food preparation [45]. Additionally, many authors showed that heat denaturation of BoNT is biphasic in time, with most activity destroyed rapidly, but with an additional heat resistant fraction (1-5 %) that is inactivated more slowly [46]. Therefore, the U.S. Center for Disease Control and Prevention (CDC) recommends boiling food for 10 min to ensure complete inactivation [47]. The most common source of foodborne botulism are home-made preserved foods that have not been correctly processed or preserved and that are not heat treated before consumption. The first signs of botulism appear typically between 12 to 36 hours after toxin ingestion, although the onset time can range from a few hours to as late as ten days depending on the dose. Symptoms include blurred or double vision, dry mouth, difficulty in swallowing and speaking, drooping eyelids, paralysis of face muscles, nausea, vomiting, descending bilateral flaccid paralysis and generalized muscle weakness. If left untreated, this may lead to fatal respiratory failure and cardiac muscle paralysis [48]. It is estimated that as little as 30-100 ng BoNT (3000 MLD_{50}) is sufficient to cause foodborne human botulism [49,50].

Besides foodborne botulism, where food contaminated with pre-formed toxin is the cause of disease, other forms have been described. Infant botulism occurs when clostridial spores are ingested and subsequently colonize the gut and release toxin *in situ*. Particularly infants under one year of age are at risk for this form of botulism because they lack a mature gut microbiota that can prevent spore germination and outgrowth. Although there are several possible sources of infection for infant botulism, spore-contaminated honey has been associated with a number of cases [51]. The estimated incubation period for development of infant botulism is three to thirty days after exposure of spores [52]. Typical symptoms are constipation, floppy movements due to muscle weakness, weak cry, drooling, drooping eyelids, difficulty feeding, and paralysis. The youngest reported victim was only 38 h old at onset of the illness [53].

Another form of botulism, designated wound botulism, occurs when spores germinate and produce toxin in anaerobic zones of wounds or abscesses. This form is primarily associated with drug users using injection needles under unhygienic conditions. Symptoms are similar to foodborne botulism, but may take up to two weeks to appear.

Inhalation botulism does not occur naturally and is associated with accidental (e.g. in the laboratory) or intentional (e.g. bioterrorism) release of toxin in aerosols. Although the exact human lethal inhalation dose has not been quantified, extrapolation from primate studies lead to an estimate of ~0.70-0.90 µg purified BoNT/A [8]. Contrary to oral poisoning, inhalation poisoning does not benefit from the presence of auxiliary proteins. Transcytosis experiments demonstrated that purified BoNT/A is actively transported from the apical to the basolateral side of polarized alveolar epithelial cells [54]. Following inhalation, symptoms become visible between one to three days, with longer onset times for lower levels of toxin. Symptoms proceed in a similar manner as described above.

Finally, some very rare cases of *C. botulinum* intestinal overgrowth have been reported of in adults with surgically altered gastrointestinal tracts. Also, injection-related botulism can occur after injection of licensed or unlicensed toxin for therapeutic or cosmotic purposes (such as Botox®). An estimated human lethal dose of 0.09-0.15 µg was reported, when crystalline BoNT/A was introduced intravenously [8].

In the U.S., 161 cases of botulism were laboratory-confirmed and reported to CDC in 2014. Foodborne botulism accounted for 9 %, infant botulism for 80 %, wound botulism for 10 % of the cases, and botulism of unknown or other etiology for 1% [55]. In Europe, 133 botulism cases including three deaths were reported to the European Centre for Disease Prevention and Control (ECDC) in 2015. The average case fatality rate in Europe from 2010 to 2015 was 5.4 % [56].

1.5.2 Treatment

Early diagnosis of botulism is very important to maximize the chances of complete and fast recovery. Administration of antitoxin should not be delayed while awaiting laboratory confirmation because the antitoxin does not reverse the disease or the existing paralysis, but only halts its progression by binding toxin that is still in the blood circulation. Patients given a trivalent antitoxin (towards toxin types A, B, E) within the first 24 hours after symptom onset had shorter hospital stays, shorter duration of ventilatory support, and a lower fatality rate of 10 % than those given antitoxin more than 24 hours after onset (15 %) or those who did not receive antitoxin at all (46 %) [3]. Since 2013, a heptavalent (A-G types) equine antitoxin was approved by the U.S. Food and Drug Administration (FDA). BabyBIG® is used to treat infant botulism, and consists of human-derived neutralizing antibody to A and B type toxins.

In case of severe botulism accompanied by respiratory failure, the patient requires not only a mechanical ventilator but also intensive care for weeks or even months. The paralysis reverses only slowly over several weeks because nerve axons have to be regenerated.

1.5.3 The use and abuse of BoNT

Despite its extreme lethal potency, BoNT is used in a wide range of therapeutic and cosmetic applications. In 1989, Botox® (BoNT/A) was approved by the FDA as an orphan drug for the treatment of (i) a disorder in which both eyes do not line up in the same direction (strabismus), (ii) hemifacial spasms and (iii) abnormal contraction or twitch of the eyelids. In 2002, approval was granted for the cosmetic use of Botox® for temporarily reducing glabeller forehead frown lines. The range of applications has further expanded since then, and Botox® has now proven valuable in the management of a wide variety of medical conditions, especially strabismus and focal dystonias, hemifacial spasm and various spastic movement disorders, headaches, hypersalivation, and other chronic conditions that only partially respond to medical treatment. The cosmetological applications include correction of lines, creases and wrinkles all over the face, chin, neck, and chest to treatment of dermatological conditions such as hyperhidrosis. [57]

In Europe, BoNT/A is marketed by another company as well, under the brand name Dysport® (Speywood, United Kingdom). One unit of Botox® has a potency that is approximately equal to four units of Dysport®. Doses of all commercially available BoNTs are expressed in terms of units of biological activity. One unit of toxin corresponds to the median intraperitoneal lethal dose (LD50) in female Swiss-Webster mice. [58]

Due to its high toxicity, BoNT can also be abused as a biological warfare weapon or for terrorist attacks. One gram of crystalline toxin, evenly dispersed and inhaled, could theoretically kill more than one million people. In reality, technical factors make such even dissemination difficult and unpredictable. Attempts have been made to use BoNT as a weapon for terrorist attacks at multiple sites in Japan, but they failed, possibly because of faulty microbiological techniques or deficient aerosol-generating equipment. Several countries, including the US, Russia, Iran, Iraq, North Korea and Syria have developed BoNT weapons in the past, and in some countries these programs may still be ongoing. [8]

1.5.4 Laboratory safety

Despite the high toxicity of BoNT, *C. botulinum* and other BoNT producing clostridia are graded as biosafety class II pathogens in Europe because they are noninvasive and noncontagious. Therefore, biosafety level II facilities and trained personnel are a minimum requirement. However, activities with high potential for aerosol formation or production of large amounts of toxin, require specific biosafety level III working procedures. In addition, access to the strains needs to be restricted because of the bioterrorism threat. [59]

1.6 A closer look into the heterogeneous species *C. botulinum*

The species *C. botulinum* is distinguished from other clostridia mainly based on its ability to produce BoNT. However, it is a physiologically and genetically heterogeneous species that is divided into four distinct groups, designated as groups I-IV. Fig. 1.3 illustrates the genetic diversity of the groups in a dendogram generated from *16S rRNA* sequences of different clostridial species.

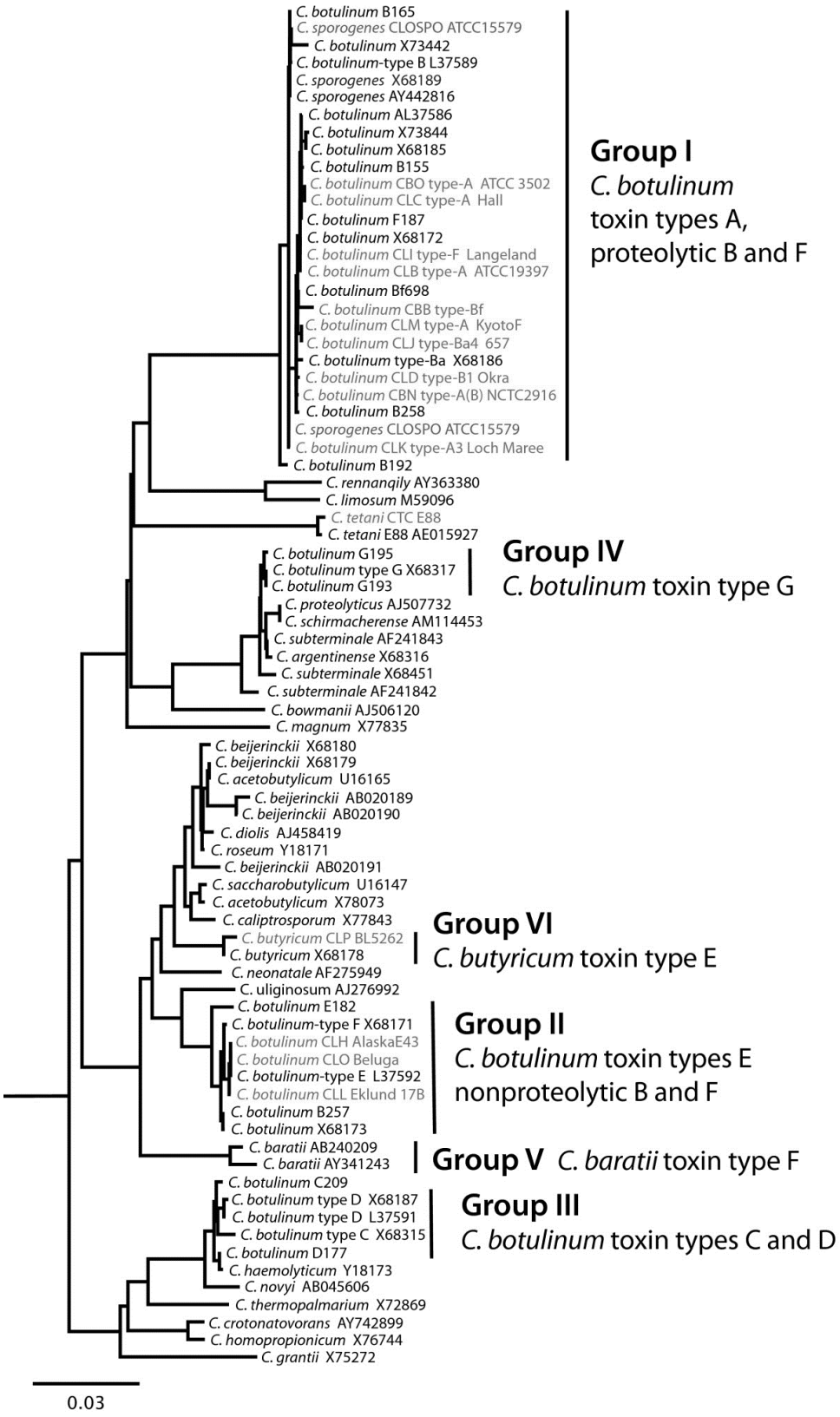

Fig. 1.3: Relatedness of *C. botulinum* and some related species in a dendogram based on *16S rRNA* sequences. Groups I-IV of *C. botulinum* are indicated, as well as group V *C. baratii*, and group VI *C. butyricum* strains. BoNT types B and F can either be produced by proteolytic group I as well as nonproteolytic group II strains. The scale of 0.03 represents 3 % diversity between sequences. Source: [60]

Group I strains (gICb), also designated as the proteolytic strains, are mesophilic bacteria with an optimum growth temperature of about 37 °C and a minimum growth temperature of 10-12 °C (Table 1.1). They can grow down to a pH of 4.6 and a water activity of 0.93. gICb strains form spores with high heat resistance ($D_{121°C} \sim 0.21$ min) and are the main target for the "botulinum cook", the process used in canning of low acid foods (121 °C/3 min or equivalent, in order to achieve at least a 12-D reduction). Because of its strong physiological similarity and close genetic relationship to gICb, *C. sporogenes* strain PA 3679 has been widely used as a safe surrogate to study the effectiveness of food preservation processes [61,62]. Recent phylogenetic analysis of whole genomes showed that the two species separate into two discrete clades with 93 % average nucleotide identity, supporting their distinction as two species [63]. Moreover, in the same study it was demonstrated that several BoNT/B producing strains, previously classified as gICb, appear more like *C. sporogenes* strains with an acquired *bont* gene. Similarly, the presence of strains lacking *bont* genes in the *C. botulinum* clade suggests the possibility that these strains were *C. botulinum* that have lost their toxin genes (which are often located on a plasmid in type B strains) and were therefore characterized as *C. sporogenes*. Furthermore, it was shown recently that *C. sporogenes* ATCC 15579, which belongs to the *C. sporogenes* clade based on average nucleotide identity, has a different set of germinant receptor genes and different germination behavior from *C. botulinum* ATCC 3502 [64]. Therefore, the use of *C. sporogenes* as a safe surrogate for gICb may not always be warranted.

Group II *C. botulinum* (gIICb) comprises nonproteolytic strains producing toxin types B, E or F, which are saccharolytic and psychrotrophic with an optimum growth temperature of 30 °C and a minimum growth temperature of 3 °C. Their spores are less heat resistant than those of gICb ($D_{82.2°C} \sim 2.4$ min), even in the presence of lysozyme ($D_{82.2°C} \sim 231$ min), which strongly increases the recovery of survivors of gIICb spores (explained in more detail in 1.7). gIICb strains can grow down to pH 5.0 or water activity 0.97 (with NaCl as a solute). In the food industry, gIICb is a major concern for the safety of minimally processed chilled ready-to-eat foods with an extended shelf life, because spores surviving the mild processing treatments may subsequently germinate, grow out and produce toxin during refrigerated storage [65,66]. The specific proteolytic cleavage that activates BoNT to become toxic is mediated by endogenous proteolytic enzymes of gICb strains. On the contrary, this is not the case in nonproteolytic gIICb strains, where BoNT needs to be activated by proteolytic enzymes of the host or the environment.

Table 1.1: Phenotypic characteristics of *C. botulinum* groups I-IV. +: all strains positive, v: some strains positive, -: all strains negative, NR: not reported. [67–70]

	Group I	**Group II**	**Group III**	**Group IV**
Neurotoxins formed	A, B, F [a]	B, E, F	C, D	G
Ferment glucose	+	+	+	-
Ferment maltose	v	+	v	-
Ferment fructose	v	+	v	-
Ferment mannose	-	+	+	-
Proteolysis [b]	+	-	-	+
Lipase production	+	+	+	-
Lecithinase	-	-	-	-
Minimum growth temperature	12 °C	3 °C	15 °C	NR
Optimum growth temperature	37 °C	30 °C	37 °C	37 °C
Minimum pH for growth	4.6	5	NR	NR
NaCl concentration preventing growth	10 %	5 %	NR	NR
Minimum water activity for growth with NaCl	0.94	0.97	NR	NR
Minimum water activity for growth with glycerol	0.93	0.94	NR	NR
Spore heat resistance [c]	$D_{121°C}$ = 0.21 min	$D_{82.2°C}$ = 2.4 min = 231 min [d]	$D_{104°C}$ = 0.1-0.9 min	$D_{104°C}$ = 0.8-1.1 min
Non-neurotoxigenic equivalent clostridia	*C. sporogenes*	No species name assigned	*C. novyi*, *C. haemolyticum*	*C. subterminale*

[a] More than one toxin type may be formed; [b] proteolysis denotes an ability to degrade native proteins; [c] D-value in phosphate buffer pH 7; [d] Decimal reduction time (D-value) without/with lysozyme in recovery medium.

Group III strains produce BoNT/C and /D and are responsible for the majority of botulism outbreaks in animals. These strains are mostly nonproteolytic, have an optimal growth temperature around 37 °C and a minimum growth temperature of 15 °C. The spores have an intermediate heat resistance ($D_{104°C}$ ~ 0.9 min) [59]. Studies have shown that group III is genetically related to the nontoxic *C. novyi* and *C. haemolyticum* (based on *16s rRNA* and whole genome analysis) [71,72]. *C. botulinum* strains producing type G toxin belong to group IV, sometimes designated as a different species, *C. argentinense*. These strains are proteolytic and asaccharolytic and differ from other groups in not producing lipase. They ferment amino acids and convert these into organic acids including acetate, butyrate, iso-valerate and phenylacetate. *C. argentinense* grows optimally at 37 °C and their spore heat resistance is similar to group III ($D_{104°C}$ ~ 0.8-1.12 min). *C. subterminale* is the nontoxigenic phenotypic counterpart of BoNT/G producing strains.

1.7 Control of group II *C. botulinum* in modern food processing

The causative agents of human foodborne botulism are gICb and gIICb strains. Since both groups have very different properties (Table 1.1), they present different risks in foods. Spores of gICb strains have a very high heat resistance and because of the severity of the toxin, they are the main safety target for processing of low acid shelf-stable foods. gICb strains are not a significant concern in acidic (pH < 4.6) or reduced moisture products (a_w < 0.93) because they are not able to grow and produce toxin in these conditions. Foodborne botulism cases associated with gICb strains are therefore mostly caused by inadequately processed home-preserved foods, such as cured meat, canned vegetables and fermented fish products. Commercial canned products rarely cause botulism because commercial processes are well designed to deliver a 12-D reduction of this pathogen, and because the processes are well controlled. However, commercial pasteurized low acid products like mascarpone and carrot juice have been implicated in botulism outbreaks due to failure to respect proper refrigerated storage conditions and/or shelf life. [49,73,74]

As noted earlier, gIICb is a major concern for the safety of refrigerated processed foods of extended durability (REPFEDs). Guidelines for control of this hazard in the food industry have been developed more than two decades ago, requiring either a 6-D reduction of the spores by heat treatment and no possible recontamination, or control of spore outgrowth by limitation of the refrigerated storage time to < 10 days, inclusion of ≥ 3.5% NaCl, ≥ 100 ppm nitrite, acidifying to pH ≤ 5.0, water activity < 0.97, or an appropriate combination of these or additional hurdles. Table 1.2 shows examples of foodborne botulism outbreaks due to gIICb strains in commercial foods since 1985. It is rare that commercially produced REPFEDs are the cause of botulism, but when it happens, it is mostly due to temperature and/or time abuse during storage. [75–77]

Table 1.2: Foodborne botulism cases due to gIICb strains in commercial foods from 1985-2016.

Country	Year	Number of cases (deaths)	BoNT type	Vehicle food item	Reference
Egypt	1991	91 (18)	E	Salted mullet fish	[78]
Germany	1998	4	E	Smoked salmon trouts	[79]
Japan	1998	6	B	Salted olives	www.promedmail.org [c]
Russia	1999	72	ND [a]	Fish	www.promedmail.org
Morocco	1999	11 (1)	B [b]	Mortadella sausage	[80]
Poland	2001	7	ND [a]	Canned fish	[81]
Alaska	2002	8	E	Whale	[82]
France	2003	4	B	Beef and poultry sausages, 'halal'	[83]
Canada	1985-2005	70 (5)	E	Seal meat and fat	[84]
Canada	1985-2001	19 (3)	E	Salmon eggs	[84]
Switzerland	1993-1994	12	B [b]	Cured ham	[85]
U.S.A.	2014	3 (1)	E	Fermented fish heads	www.promedmail.org
Ukraine	2015	7 (2)	ND [a]	Dried fish	www.promedmail.org
Spain and Germany	2016	2 in Spain 4 in Germany	E	Dried salted roach	www.promedmail.org

[a] ND, no data available ; however, botulism related to fish products is frequently due to BoNT/E.

[b] The genetic group of BoNT/B producing *C. botulinum* was not reported.

[c] "ProMED : the Program for Monitoring Emerging Diseases - is an Internet-based reporting system dedicated to rapid global dissemination of information on outbreaks of infectious diseases and acute exposures to toxins that affect human health."

To achieve a 6-D reduction, a treatment of 90 °C/10 min or equivalent has been proposed in the 1990's by the Advisory Committee on the Microbiological Safety of Food, and has long been widely accepted. In general, spore heat resistance varies considerably depending on the spore history, the heating medium and its pH, water activity, protein and fat content, and also shows important strain to strain variation. In addition, the presence of lysozyme or related enzymes in the food is known to increase the number of heat treated survivors of gIICb spores. Without lysozyme in the recovery medium, gIICb spores are rapidly inactivated at temperatures above 80 °C, because the cortex hydrolases which are required for spore germination are heat sensitive and thus germination and outgrowth is abolished. However, when exogenous lysozyme is incorporated in the recovery medium/food, this enzyme can substitute the role of the cortex hydrolases, resulting in a much higher fraction of survivors at high temperatures. In addition, the existence of a lysozyme-impermeable and -permeable fraction in a spore population is well described, and it is estimated that the latter represents 0.02-3.1% of the spore population [86–88]. As a result of this, heat inactivation of gIICb spores in the presence of lysozyme typically follows a biphasic course. Wachnicka et al. (2016) recently

did a systematic assessment of the heat resistance of gIICb spores based on 753 D-values and 436 z-values extracted from literature [88]. Based on their results, summarized in Fig. 1.4, they concluded that the time required for a 6-D reduction in spore load at 90 °C is ~5 min in the absence of lysozyme, indicating that the current guidance provides a suitable level of safety. However, they additionally concluded that in the presence of lysozyme, ~80 min at 90 °C may be required to achieve a 6-D reduction. This study therefore calls for a re-evaluation of the existing safety guideline with regard to psychrotrophic *C. botulinum*. This is particularly important in view of the trend towards further reduction of intrinsic hurdles (salt, preservatives, pH) while maintaining or increasing shelf life. Furthermore, innovations like the use of natural food preservatives and nonthermal processing methods such as high pressure or pulsed electric field treatment, will require new studies to investigate their effectiveness to eliminate or control gIICb. Finally, also the use of novel ingredients or food raw materials, (e.g. various alternative protein sources like insects and algae, non-acidic juices and beverages) may have an effect on the gIICb risk, for example because some of these sources may contain lysozyme, and should therefore be investigated.

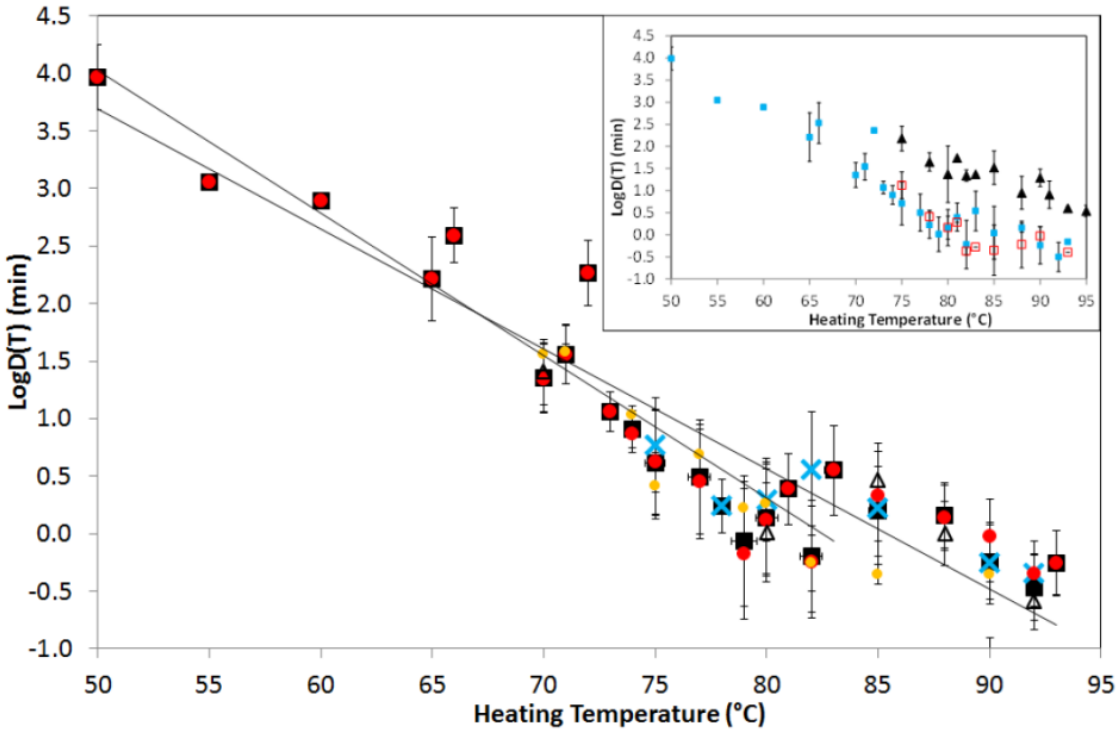

Fig. 1.4: Summary of D-values of gIICb spores extracted from literature sources. Data points and error bars correspond to mean +/- standard deviations of all D-values found at a particular temperature in the absence of lysozyme. Black squares correspond with all the data and blue crosses, red circles, orange circles and open triangles correspond with B, E and F and mixed toxin types, respectively. The solid lines represent the best fit to experimental data in the temperature range from 50 °C to 83 °C and for the whole data set, respectively. The inset graph compares D-values measured in the absence of lysozyme (blue closed squares), and in the presence of lysozyme (heat sensitive fraction = red open squares; heat resistant fraction = closed triangles). Source: [88]

Chapter 2

Sporulation and germination

2.1 Sporulation

Bacterial endospores, hereafter called spores, are dormant cellular structures that in some cases can survive for thousands or even millions of years with little or no metabolic activity, and return to active vegetative cell division in only a few minutes [89]. In the dormant form, spores are highly resistant to conditions such as heat, salinity, acidity, radiation, oxygen (depletion), low availability of water or nutrients [90–92]. Spore-forming bacteria thus ensure their future under harsh conditions by forming dormant spores. Despite being dormant, spores have a system for sensing specific nutrients in the environment that serves to activate the spore germination mechanism and return to the metabolically active vegetative state. In order to preserve the potential for viability, spores have to prevent damage to the critical cellular components needed for germination and outgrowth, or have to repair those damaged components during germination.

Spore-forming bacteria are widespread in different metabolic groups of the low-GC Gram-positive bacteria (*Firmicutes*): aerobic heterotrophs (e.g. *Bacillus* and *Sporosarcina* spp.), microaerophilic lactate fermenters (e.g. *Sporolactobacillus* spp.), anaerobes (e.g. *Clostridium*, *Moorella*, *Thermoanaerobacterium*, *Caloramator*, *Oxobacter* and *Anaerobacter* spp.), sulfate reducers (e.g. *Desulfotomaculum* spp.) and even phototrophs (e.g. *Heliobacterium* and *Heliophilum* spp.). However, the sporulation process has been studied in most detail in *Bacillus*, more specifically *B. subtilis* and *B. cereus*, and to a lesser extent in *Clostridium*. [93]

2.1.1 The sporulation process

When environmental conditions become unfavorable, spore-forming bacteria can initiate the sporulation process, a complex cascade of events that is strictly regulated at the transcriptional and post-translational level. *B. subtilis* has been used extensively as a model to study this process, and although sporulation proceeds via the same basic morphological stages in other sporulating bacteria including clostridia, recent studies have indicated major differences in regulation. Even the trigger that initiates sporulation is under debate in clostridia. While nutrient deprivation has long been considered as the universal trigger in all sporulating bacteria, recent evidence points to accumulation of the fermentation end products butyrate and acetate in *C. acetobutylicum* to cause initiation of sporulation, even in excess of nutrients. In general, a decline in growth rate leads to onset of sporulation, but in contrast to what is the case for bacilli, clostridia need an exogenous supply of energy (possibly because anaerobic metabolism is less efficient). Different stresses, for example oxygen exposure, could therefore play a role in initation. [94–96]

The different stages of sporulation are shown in Fig. 2.1 [96]. Stage 0 represents the vegetative state, undergoing normal binary fission. Before the onset of sporulation or in stage I, cells undergo a transition that is unique to clostridia, into what is known as the 'clostridial form'. Cells in this stage are swollen and accumulate vesicles containing components such as polysaccharides or polyhydroxybutyrate. In *C. saccharobutylicum* this polysaccharide was designated granulose, while in *C. botulinum* (type E) accumulation of the polymer amylopectin was demonstrated [97]. However, it should be noted that the production of these spore-associated polysaccharides has not been confirmed or further studied since it was first reported in the 1970s. Stage II is when the cells divide asymmetrically, which is the first clear indication that the sporulation process has started. The smaller compartment that is initially separated by a septum, subsequently gets engulfed by the mother cell in stage III. This happens in a way similar to phagocytosis, creating a cell within a cell. In stage IV peptidoglycan (PG) is deposited between the membranes of the mother cell and the prespore. The PG wall is composed of an inner layer, the germ cell wall, which is synthesized first, followed by the outer layer, the cortex, that consists of modified PG with very little cross-linkage. The germ cell wall and cortex are synthesized from within the prespore and the mother cell, respectively [98]. Subsequently, spore coat and cortex proteins, synthesized by the mother cell, are deposited after which the mature spore is released by lysis of the mother cell (stage V-VII).

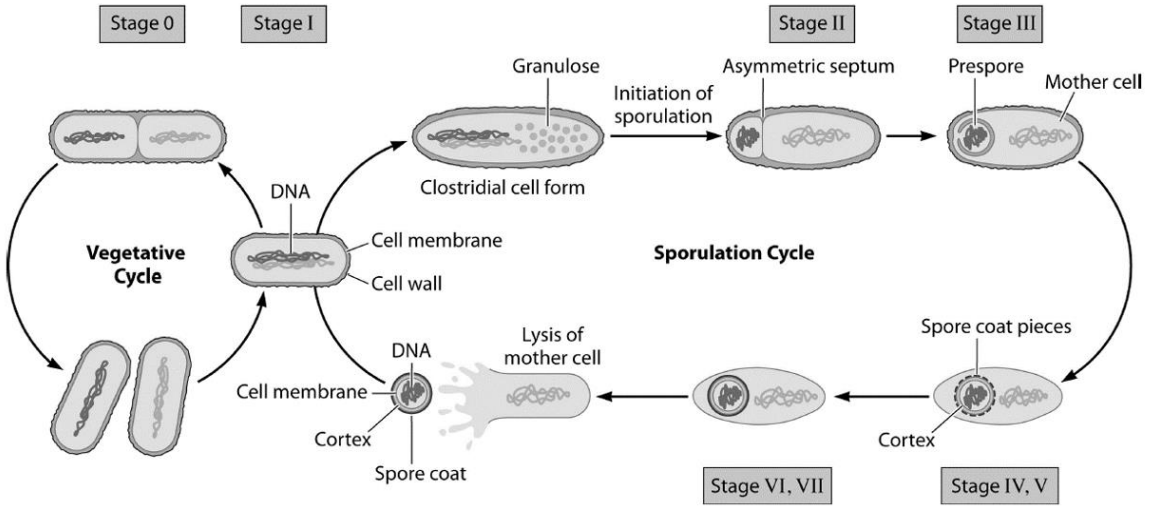

Fig. 2.1: The sporulation model of clostridia. Adapted from [96]

Although the complex sporulation process was first assumed to be well conserved, substantial differences in the sporulation genes and their regulation have been found between bacilli and clostridia in recent years. Sporulation in *Bacillus* begins with a phosphorelay, involving five histidine kinases, of which the last one phosphorylates Spo0A, the master regulator of sporulation. In contrast, clostridia do not possess this phosphorelay, but Spo0A is phosphorylated directly by an orphan histidine kinase. Other differences are found downstream of Spo0A, in the mode of activation and function of sporulation sigma factors (σ^F, σ^E, σ^G, and σ^K), not only compared to *Bacillus* but also among *Clostridium* species. Recent studies showed that σ^K, the last sigma factor that is activated in the *B. subtilis* model, is involved in the early stages of sporulation in *C. acetobutylicum*, *C. perfringens*, and *C. botulinum* as well as in the late stages of spore maturation in *C. acetobutylicum* [96]. For a more detailed discussion of the cellular mechanism and regulation of sporulation in clostridia and the differences with bacilli, we refer to Al-Hinai *et al.* (2015) and Dürre (2014) [95,96].

2.1.2 Spore structure and spore resistance characteristics

The specific physiological features of bacterial spores can be explained by their unique structure and by the presence of some unique constituents. As shown in Fig. 2.2, spores consist of a dehydrated core surrounded by different protective layers. From inside to outside, the core, the inner membrane, the germ cell wall, the cortex, the outer membrane, the coat and the exosporium can be distinguished [91].

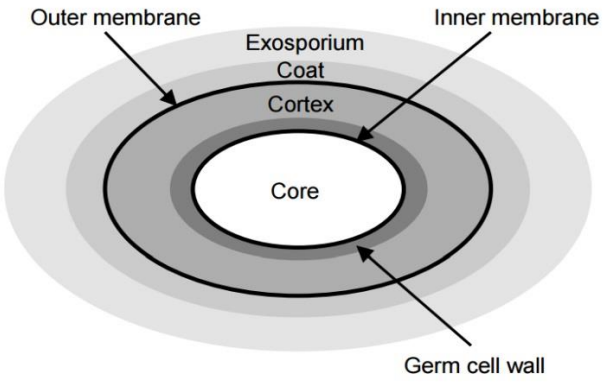

Fig. 2.2: Schematic overview of bacterial spore structure. The various spore layers are not drawn to scale, and sizes of the various layers vary significantly between spores of different species. Source: [91]

The **spore core** is derived from the mother cell protoplast and forms an environment that stabilizes DNA, RNA and enzymes, mainly because it is dehydrated. It contains minimal levels of common high-energy compounds, such as ATP and other nucleoside triphosphates. In contrast, AMP and ADP are present in significant amounts, as well as 3-phosphoglyceric acid, a potential rapid source of ATP [99]. Further, the core contains two unique macromolecules,

pyridine-2,6-dicarboxylic acid or dipicolinic acid (DPA), and small acid soluble proteins (SASPs). DPA is not produced by vegetative cells and comprises 5-15 % of the dry weight of both *Bacillus* and *Clostridium* spores, where it occurs mostly as a 1:1 chelate with Ca^{2+} and other divalent cations. By replacing core water, the large depot of DPA contributes to a low water content (25-50 % of wet spore weight) and as such it plays a role in the spore's resistance to wet heat. The low water content and activity is believed to be the major factor in the spore's enzymatic dormancy and wet heat resistance. Unfortunately, the mechanism for water content reduction during sporulation is not known. A remarkable side effect of DPA accumulation is that it decreases UV resistance, because DPA acts as a strong photosensitizer [90,100,101].

The second type of macromolecules that plays an important role in spore resistance are the SASPs, small proteins that comprise 7-20 % of the total spore protein. Two types of SASPs are distinguished based on their primary sequence. α/β-type SASPs (molecular weight 5 – 7 kDa) are found in *Bacillus* and *Clostridium*, and up to seven variants can be present in a spore. γ-type SASP (molecular weight 8 – 11 kDa) appears to be present in *Bacillus* only, and only a single variant is normally produced. The α/β-type SASPs are basic proteins that nonspecifically bind the spore DNA and alter its structure and properties. As a result the DNA is protected against damage by heat, UV radiation and chemicals. When the spore germinates, the SASPs are degraded and serve as a source of amino acids during reinitiation of protein synthesis. γ-SASP does not bind DNA and thus doesn't play a role in DNA protection. This SASP solely acts as a reservoir of amino acids during germination. [91,102]

The spore core is confined by the **inner membrane**, which is the major permeability barrier for entry of molecules into the core. Although the lipid composition of this membrane is similar to that of a vegetative cell, its water permeability is three to four orders of magnitude lower. It is noteworthy though that the membrane is by no means impermeable to water and some other small solutes, and spores in an aqueous environment, despite their dehydrated core, exchange water with the environment at a timescale of hours [103,104]. The reduced permeability has been attributed to the fact that the majority of lipids in the inner membrane is immobile. One possible explanation for this is that the cortex is a rigid shell that compresses the membrane during sporulation. During spore germination, the cortex hydrolases are activated and cause a relaxation of the pressure exerted by the cortex, which leads to water uptake into the core. As a result the volume that the membrane encloses increases up to twofold without new membrane synthesis, and this normalizes lipid mobility and membrane permeability. [105,106]

The **germ cell wall** is located between the inner membrane and the cortex, and serves as a primer for vegetative cell wall assembly during outgrowth. It is composed of PG with a similar structure to that of vegetative cells. The **cortex** also consists of PG, with the same basic architecture but with some distinguishing features. The shared basic architecture consists of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues which are coupled by a β-1,4-glycosidic bond. All NAM residues have short peptide side-chains of which some form peptide cross-links to peptide chains on adjacent NAG-NAM glycan strands. However, the degree of cross-linking is significantly reduced in cortex PG, which can be attributed to two main changes. First, 50 % of the NAM residues are converted to muramic-δ-lactam (MAL) and thus have no peptide side chains. This MAL also serves as a recognition signature for cortex lytic enzymes, which selectively hydrolyze the cortex PG while leaving the germ cell wall intact. Secondly, 25 % of the remaining NAM residues have their peptide chains cleaved to a single L-alanine residue, preventing them to participate in cross-linking. [107,108]

The cortex is surrounded by the **outer membrane**. Although it is essential in spore formation, there is discussion on whether this membrane remains intact in the mature dormant spore because it is difficult to observe it with electron microscopy. There are also no reports of the isolation of a purified outer membrane. The outer membrane lipid composition is probably similar to that of a vegetative cell membrane, since it is a remnant of the mother cell. The outer membrane is believed not to be an important permeability barrier or to have any important influence on spore properties. [90,93,109]

A next structure that is crucial for spore resistance is the **spore coat**, a layer composed of at least seventy different proteins in *B. subtilis* that are produced by the mother cell and begin to localize to the spore surface during engulfment. Bioinformatic evidence indicates that about half of the known *B. subtilis* coat protein genes have recognizable orthologues in other *Bacillus* species, whereas the other half do not appear to be conserved at all. In the genomes of *Clostridium* spp., conservation of *B. subtilis* coat genes is even more limited, with only eighteen orthologues identified in *C. difficile* [110,111]. It has been speculated that diversity in coat composition may reflect ecological niche adaptation of the spore-former [111], although no clear associations have been reported so far. The coat acts as a shield that restricts access of harmful molecules to the vital structures of the spore in two different ways. First, it acts as a permeability barrier to large molecules, e.g. lytic cortex-degrading enzymes. In addition, the coat contains enzymes that detoxify oxidizing agents such as peroxides, nitrous acid, halogens, and aldehydes. However, the coat seems to be unable to protect the spore from

certain other small toxic chemicals like alkylating agents. In addition, spore germinants can penetrate through the coat and cortex to reach the germinant receptors that are located in the inner membrane. [93,106,109]

The outermost structure of the spore is **the exosporium**, although it is not present in all spores. The exosporium layer has been studied in detail in *B. anthracis*, *B. cereus* and *B. thuringiensis* and recently also in *C. sporogenes* spores [112,113]. In these species it is described as a sac-like structure which consists of a basal layer surrounded by an external nap of hairlike projections, and it likely contributes to adhesion, dissemination, and sometimes virulence.

2.2 Spore germination

When conditions become favorable, spores exit their dormant state and return to active growth through a complex process called germination. This process is of great importance in food microbiology, because only upon germination a spore can become a vegetative cell that can cause food spoilage or poisoning. Germination is induced when specific environmental nutrient germinants are sensed by the spore germinant receptors. After binding of these nutrients, a signal transduction pathway is activated that triggers a self-propagating and irreversible cascade of events, starting with the release of monovalent cations (H^+, Na^+, K^+) and Ca^{2+}-DPA from the core, followed by degradation of the spore cortex leading to core rehydration and eventually to reinitation of metabolism and outgrowth. This process is accompanied with the loss of the spores' resistance properties. The presence of oxygen has no effect on the initial steps of *Clostridium* spore germination, reflecting that the process is independent of active metabolism. However, oxygen inhibits subsequent outgrowth.

Besides the classical germinants also other inducers have been described, termed non-nutrient germinants, e.g. exogenous Ca^{2+}-DPA, cationic surfactants like dodecylamine, muropeptides, and even high pressure. Unfortunately most of these studies were performed with *Bacillus* spores, emphasizing the need for more research in *Clostridium*.

2.2.1 Nutrient induced germination

2.2.1.1 The Ger family of receptors

Germination is naturally induced by nutrient germinants binding to germinant receptors (GRs) that are located in the spore's inner membrane. These germinants are species and strain specific, and the most common include L-amino acids, purine bases and D-sugars. The most widespread GRs are the Ger-type receptors, and these have long been believed to be universal to all spore-forming bacteria. These GRs are typically composed of three protein subunits (designated A, B and C), often encoded in a tricistronic operon. The first Ger receptor identified in *B. subtilis*, which binds to L-alanine, was designated GerA and its three subunits as GerAA, GerAB and GerAC. Besides GerA, *B. subtilis* contains two additional Ger receptors named GerB and GerK. These two receptors act cooperatively to trigger germination with either L-asparagine supplemented with glucose, fructose, and potassium ions (GFK) or L-alanine supplemented with GFK. The number of Ger receptors varies between genera, species and even strains, and some strains have up to seven different Ger-type receptor operons [114]. Because more and more receptors are being identified, Ross & Abel-Santos (2010) addressed a problem regarding the inconsistency in nomenclature of the Ger receptors [115]. In many cases,

GR names were designated randomly, without consideration for phylogenetic relationships or functionality. The new proposal attempts to remediate this issue by introducing a systematic nomenclature, based on phylogeny and functionality. Unfortunately, they only addressed Ger receptors in bacilli, because they found that there is not yet enough genomic and functional information of receptors in the *Clostridium* genus. In *C. botulinum*, the designation GerX is sometimes used because the cognate germinant(s) of the encoded receptors is/are still unknown.

The A and B subunits of Ger receptors are both predicted to be integral membrane proteins. The A protein consists of four to six predicted membrane-spanning domains, as well as large N and C terminal hydrophilic domains. The B protein contains seven to twelve transmembrane helices, and despite low sequence homology, its predicted tertiary structure resembles that of a superfamily of membrane-associated single-component membrane transporters. The C subunit is predicted to be a lipoprotein that is anchored via a diacylglycerol unit to the outer surface of the spore inner membrane [90]. It was recently suggested that subunit B is responsible for germinant binding in *B. megaterium* [116,117]. In this study, a nongerminating mutant was complemented with various plasmid-borne mutant receptor genes, revealing amino acid residues that may be directly involved in germinant binding. Unfortunately, the fact that two GR subunits are integral membrane proteins has hampered their purification, and thus the study of their biochemical properties.

Many different GR loci have been described next to the *gerABC* architecture, especially in clostridia [118,119]. Some receptor subunits are encoded as orphan monocistronic genes, while some operons encode duplicated subunit genes. Brunt et al. (2016) recently reported on the variety of genomic Ger clusters in groups I-IV of *C. botulinum*, based on *in silico* analysis of 156 *C. botulinum* and *C. sporogenes* genomes [120]. The latter were included because of their close relatedness to gICb. Four different *gerX* clusters were identified, as shown in Fig. 2.3 D. These clusters could be further separated into six subtypes for cluster *gerX1* (ABC configuration), three subtypes for *gerX2* ((AB)ABCB configuration), two subtypes for *gerX3* (CA-B bicistronic configuration) and one single type *gerX4* (ACxBBB configuration). The x putative subunit in the latter is a hypothetical protein of which the functionality is unknown so far. The similarity within the subtypes of GerX1 and GerX3 is more than 90 % (except for GerX1c, 85 %), while the similarity between subtypes of different groups is 20 – 65 %.

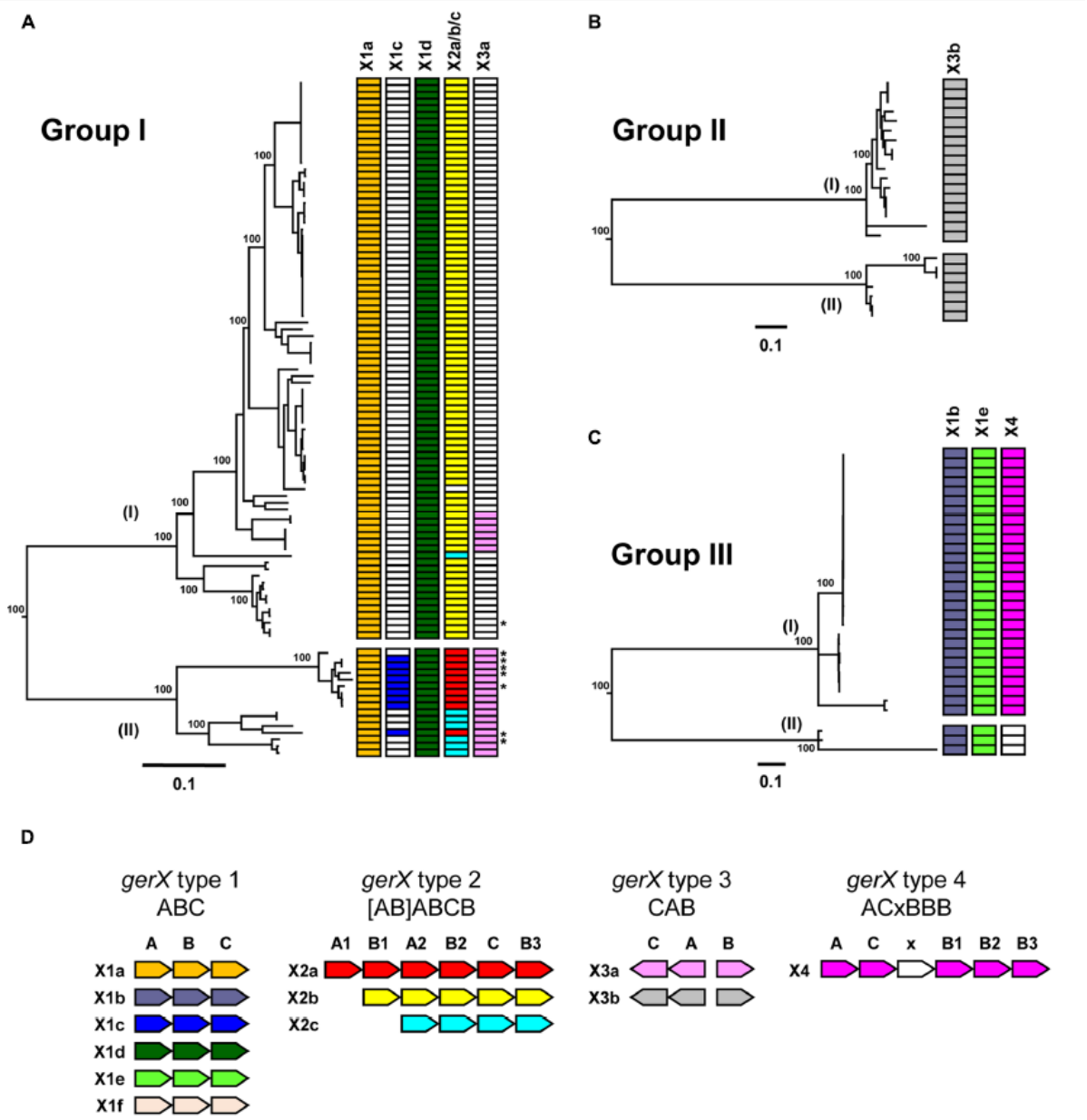

Fig. 2.3: Distribution of Ger receptor gene clusters in *C. botulinum* and *C. sporogenes*. The phylogenetic trees in A-C are based on single nucleotide polymorphisms as determined using ParSNP. Values shown at branches represent bootstrap values provided by ParSNP. (A) shows the Ger cluster distribution in *C. botulinum* group I and *C. sporogenes* genomes where the latter is indicated with asterisks, (B) shows the distribution in *C. botulinum* group II and (C) in group III. The colors in trees (A-C) correspond with the colors attributed to each individual *ger* subtype shown in (D). White blocks represent absence of the specific GR subtype. (D) Genetic organization of the *ger* subtypes. *ger1f* is only found in *C. botulinum* group IV which is not shown in (A-C). Adapted from [120]

gICb and *C. sporogenes* strains typically encode three to five different Ger receptor subtypes, and this may relate to their ability to germinate by various amino acids, often in combination with L-lactate, although the latter is not always essential [64,121]. The functionality of Ger receptors in *C. botulinum* has been experimentally demonstrated only in one gICb strain and one *C. sporogenes* strain by construction and analysis of insertional knockout mutants [64]. Unexpectedly, although both strains contained *gerX1a* and *gerX1d* (Fig 7A), only GerX1d was essential for nutrient induced germination in *C. sporogenes* whereas both GerX1a and GerX1d

were essential in the gICb strain. Subtle differences in amino acid sequences could thus lead to different functionalities, although other (unidentified) proteins could also play a role. The same study showed that neither GerX2b or GerX2c, both also present in the gICb and *C. sporogenes* strains, could promote germination alone. Some gICb strains also have a *gerX3* locus. This locus is also present in all sequenced gIICb strains (Fig 2.3 B/D), and is organized in two transcriptional units with *gerB* transcribed in the opposite direction of *gerA* and *gerC*.

Similarly to gICb, gIICb strains respond to several amino acids, although for gIICb the presence of L-lactate is considered essential for germination. Plowman and Peck (2002) demonstrated optimal germination of three gIICb strains in L-alanine, L-cysteine or L-serine, all in combination with L-lactate [122]. Moreover, an older study had reported that germination of gIICb spores was also possible by single amino acids (without addition of L-lactate) at pH 9, as well as by the combination of L-alanine with glucose, galactose or maltose at neutral pH [123]. In large contrast to gICb strains, gIICb strains contain only one receptor, GerX3. It remains to be clarified how one receptor can respond to such a variety of nutrients.

Mutational studies in *C. perfringens*, which contains the *gerK* locus that has a similar bicistronic organization to *gerX3* led to the conclusion that GerKC is the main receptor protein involved in nutrient and non-nutrient germination (by dodecylamine and Ca^{2+}-DPA, discussed further in 2.2.2) [124,125]. In addition to the *gerK* locus, there is a monocistronic *gerAA* located rather far away from *gerK*. The fact that the A and B subunits are dispensable in *C. perfringens* is in striking contrast with the situation in *B. subtilis*, where all three subunits are essential for formation of a functional GR. The GerKC receptor reacts to germinants such as KCl, L-asparagine, or an L-asparagine–KCl mixture.

Despite the wide distribution of Ger-type receptors in sporulating bacteria, the genome sequences of at least two *Clostridium* species, *C. barletti and C. difficile*, do not contain Ger gene homologs. Nevertheless, spore germination in these bacteria is also induced by specific germinants, consistent with a mechanism involving one or more specific receptors. *C. difficile* spores do not react to amino acid germinants, but germinate in response to taurocholate, a characteristic component of bile. The use of this compound as a germinant therefore allows *C. difficile* to germinate in its primary niche, the animal gut. Francis et al. (2013) identified that the catalytically inactive serine protease CspC functions as the receptor of the germinant taurocholate in *C. difficile* [126]. This was found by analysing different germination-null mutants, of which many carried SNPs in *cspC*. The finding was further corroborated by analysis of a mutant that germinated in response to chenodeoxycholic acid, a compound related to taurocholate but that normally acts as a competitive inhibitor of germination. This mutant

contained a mutated CspC residue that was proposed to modify the bile acid binding pocket to be less stringent. It was thus suggested that CspC is able to bind bile salts, resulting in a conformational change that is transmitted to CspB (another, but active serine protease). CspB would then cleave the N-terminal part of the cortex hydrolase SleC, thereby converting the inactive zymogen into an active cortex lytic enzyme, leading to germination. Unfortunately, while many clostridia contain these Csp serine proteases, of which the encoding genes (A, B and/or C) are located directly upstream of *sleC*, it has not been studied so far if they act as GRs as well [127].

2.2.1.2 Signal transduction and cortex hydrolysis

The binding of GRs with their germinants induces an irreversible cascade of events, suggesting that a signal is transduced from the GR. Although it is currently unknown how this signal is transduced, the subsequent events are well described in *Bacillus*. First, Na^+, K^+ and H^+ ions are released from the core, followed by Ca^{2+}-DPA. In many *Bacillus* species, the released DPA then activates a cortex hydrolase. However, *C. perfringens* spores lacking DPA germinate similarly to wild-type spores, showing that DPA is not involved in the signalling cascade in this case [128]. DPA release during germination as well as DPA uptake in the developing spore during sporulation are suggested to be mediated by the SpoVA proteins, and also in this respect there are differences between clostridia and bacilli: *B. subtilis* and many other *Bacillus* species have a heptacistronic *spoVA* operon (SpoVAA, B, C, D, Eb, Ea and F, encoded in this order), whereas *C. perfringens*, *C. botulinum*, *C. difficile* and other clostridia have a tricistronic *spoVA* operon (SpoVAC, -D and –Eb). Tetra- and pentacistronic operons exist as well (e.g. in *C. asparagiforme*, *C. hathewayi*), but SpoVAC, -D and –Eb are always present [127,129]. It is not clear how GRs transduce the signal further downstream, but protein-protein interactions have been detected with some SpoVA proteins in *B. subtilis* [130]. Unfortunately the precise function of the SpoVA proteins is unknown, as is the exact organization of the channel that they form and how it works [90,131].

Although DPA release precedes cortex hydrolysis in *B. subtilis* and *C. perfringens*, it is the other way around in *C. difficile* [132]. In *C. botulinum* this is yet unknown. Cortex hydrolysis is an essential step in germination since it allows the core to further take up water, which is a prerequisite for resuming metabolism. The cortex lytic enzymes (CLEs) are the necessary players in this process. They recognize and cleave the spore cortex PG with its characteristic modification as described in 2.1.2, leaving the germ cell wall intact. These enzymes are preformed and need to be activated during germination. Two different machineries are well conserved in *Bacillus* and *Clostridium* species. The first system involves two CLEs, CwlJ and

SleB, both synthesized in a mature form during sporulation of most bacilli and some clostridia [127,133,134]. In some bacilli it has been demonstrated that either CLE alone is sufficient for completion of germination, whereas spores of a *cwlJ sleB* double mutant exhibit extremely low viability [90]. It has been shown that Ca^{2+}-DPA release activates CwlJ and that this CLE requires GerQ for proper localization to the cortex/coat boundary [135,136]. In contrast, the mechanism of SleB activation is unknown. In *Bacillus*, *sleB* is located upstream of *ypeB* in a bicistronic operon and it appears that YpeB is required for localization and/or activity of SleB [137,138,139]. In many clostridia, including gIICb, in which *sleB* is encoded, no *ypeB* gene is identified [127]. This questions the activity of SleB in these species. In contrast, gICb strains encode *sleB* as well as *ypeB*.

The second and mechanistically different CLE machinery involves SleC. This CLE is well conserved in most *Clostridium* species and has been studied in *C. perfringens* and *C. difficile*. SleC is synthesized as an inactive zymogen containing a pro-region that is cleaved by Csp proteases early in germination, converting the zymogen into an active CLE. The proteases belong to the subtilisin subfamily. To date, the mechanisms that regulate the activity of the Csp proteases are unknown. Functional studies showed that SleC is the essential cortex hydrolase in both *C. perfringens* and *C. difficile*, which additionally possess a SleM and a SleB homolog respectively. SleM is synthesized as a mature muramidase which is believed to degrade PG fragments generated by SleC during germination. In *C. perfringens*, deletion of *sleM* led to spores that germinated as wild type spores [140]. In *C. difficile*, insertional inactivation of the SleB homolog also had no effect on germination and outgrowth [141]. The lack of *ypeB* could possibly explain SleB's inactivity in *C. difficile*, but this would have to be confirmed with the construction of a double mutant *sleB sleC*.

The study of Brunt et al. (2016) that was described above for its detailed *in silico* analysis of Ger receptors in *C. botulinum*, also catalogued the CLE homologs in 156 genomes of *C. botulinum* I-IV and *C. sporogenes* [120]. All gICb and group III strains contained at least one *sleB* copy (with a single exception) as well as a single copy of *cwlJ* and *ypeB*. Meaney et al. (2015) showed that SleB and YpeB are required for optimal germination in gICb strain ATCC 3502, and that no functional CwlJ enzyme is formed although its encoding gene is present [133]. However, insertional inactivation of *cwlJ* in another gICb strain, ATCC 15579, decreased the germination rate [120].

The bioinformatic analysis additionally revealed genes in group I-III encoding four 'SleB-like' proteins, designated SleB2-5. SleB2 is found in group I, II and III and is a putative cell wall hydrolase. SleB3, found in group I and III, consists of two LysM domains which are involved

in PG-binding. The coding sequences of both proteins are preceded by a *ydaO* element, except in gIICb strains. This element is a riboswitch that is often associated with genes involved in polysaccharide degradation in *Bacillus* [142]. SleB4 is found in only three gIICb strains (CB11/1-1, 20536 and ATCC 17786), and also belongs to the cell wall hydrolase family. SleB5 is found in only one group III strain, contains two PG-binding domains and shares ~40 % amino acid identity with proteins annotated as spore cortex-lytic enzymes [120].

gIICb strains possess *sleB2*, *sleM*, *sleC*, and also other *sleC*-like genes designated as *sleC*2a/b, whereas *ypeB* and *cwlJ* appear to be absent [119,120,127]. Like SleC, SleC2a/b contains a SpoIID/LytB domain. However, there is only one PG-binding domain in SleC, compared to five in SleC2a and four in SleC2b. Thus, SleC is ~443 residues long, SleC2a is ~792 and SleC2b ~698 residues. The latter is mainly found in type E strains. The functionality of all these predicted proteins needs to be confirmed experimentally, but it is clear that there is a large diversity in CLEs in *C. botulinum* strains.

2.2.2 Non-nutrient induced germination

While nutrient germinants are considered to be the natural inducers of germination, some other agents are capable of causing germination as well. The mechanism differs from nutrient induced germination as it can bypass certain events described in 2.2.1. For example, **exogenous Ca^{2+}-DPA** can directly activate CwlJ in *Bacillus* without the involvement of GRs [135]. In contrast, GRs do play a role in Ca^{2+}-DPA germination in *C. perfringens* since spores in which the receptor GerKC was insertionally inactivated, germinated very poorly with Ca^{2+}-DPA[125]. In contrast to *B. subtilis* and *C. perfringens*, *C. difficile* spores do not germinate with Ca^{2+}-DPA although a CwlJ homolog is present (30 % amino acid identity with *B. subtilis*) [143].

Another group of non-nutrient germinants are cationic surfactants such as **dodecylamine**. Although the exact mode of action of this compound is not yet clear, one possibility is that it triggers germination by opening the spore's Ca^{2+}-DPA channels [131,144]. Vepachedu & Setlow (2007) proposed two possible mechanisms [131]. Either dodecylamine directly opens a channel in the inner membrane, or the interaction of the surfactant with the membrane causes an alteration in the membrane's properties, which in turns opens the channel [131]. In addition, it has been suggested that the formation of salt bridges between the $-NH_2$ head groups of dodecylamine and carboxylate anions in the spore cortex would destabilize the cortex and make the core unable to maintain its low water content [145]. In *B. subtilis* it was reported that dodecylamine induced DPA release occurs without the involvement of the GRs since spores lacking all GRs released DPA at a similar rate to that of wild-type spores upon exposure to

dodecylamine [144]. Observations were somewhat different for *C. perfringens*, where GerKC deficient spores released slightly less DPA than wild-type spores [125]. Dodecylamine has been shown to induce germination in *C. difficile* as well [143]. In all these studies, germination was mainly assessed based on DPA release, and it has been demonstrated that further germination is halted because spores do not swell and do not take up enough water to reinitiate metabolism [125,143,144]. This type of germination is sometimes described as lethal germination because viable spore counts are drastically reduced in the presence of this surfactant [145].

In *B. subtilis* it has been demonstrated that also **PG-derived muropeptides** can act as a germination trigger, even at extremely low concentrations (<1 pg/ml). Muropeptides are released during vegetative growth, when there is a high PG turnover with balanced degradation and synthesis activities. Consequently, muropeptides in the environment could serve as a signal for spores that conditions are favorable for outgrowth. Only muropeptides that have *meso*-diaminopimelate at position three of the stem peptide linked to the oligosaccharide backbone could induce *B. subtilis* spore germination. PG fragments from some other Gram-positive bacteria, like *Staphylococcus aureus*, contain L-lysine in the third position and are thus ineffective in inducing *B. subtilis* spore germination. The ser/thr protein kinase PrkC was shown to be essential for signal transduction in the muropeptide germination mechanism. The extracellular domain of PrkC contains PASTA (penicillin and ser/thr kinase associated) repeats that can mediate PG binding. In response to this binding, PrkC phosphorylates the essential translation factor EF-G, as it similarly does in stationary phase cells. It is suggested that the phosphorylated EF-G subsequently modulates ribosome activity and hereby stimulates translation, thereby inducing an exit from the spore's dormancy [146–148]. However, it is yet unclear how spore rehydration, cortex degradation and other essential steps in germination fit into this mechanism. Since *prkC* is strongly conserved between clostridia and bacilli, it is postulated that this germination pathway may be universal. However, muropeptide induced germination nor the role of PrkC have been documented in spore-formers other than *B. subtilis*.

Besides specific molecules, also physical treatments like **high hydrostatic pressure** (HP) can trigger spore germination. While pressures over 1000 MPa or the combination of HP and heat are required to kill bacterial spores [149], less extreme conditions can induce germination. Although this has been well studied in *Bacillus* species, less is known about clostridial spore germination by HP. In *B. subtilis*, there is a difference in the germination mechanism depending on the pressure level [100]. Moderate pressures (50-300 MPa) induce germination only in the presence of intact GRs, and thus appear to somehow activate the germination

pathway similar to nutrients. In contrast, higher pressures (400-800 MPa) seem to directly act on the Ca^{2+}-DPA channels causing DPA release, without the involvement of the GRs [150–152]. However, in the latter case germination may be incomplete because SASPs are not degraded and ATP is not produced. The reason for this may be that the HP treatment inactivates some enzymes in the core. Since α/β-SASPs are not degraded, spores remain partly resistant to an additional HP treatment, hydrogen peroxide and UV radiation, but not to moist heat [153]. When high pressure (> 500 MPa) is applied in combination with elevated temperatures (T > 90 °C), spores are rapidly killed, probably by a mechanism similar to thermal inactivation that does not involve a physiological germination mechanism. This combined treatment has potential commercial applications to produce high quality, low acid, shelf-stable foods [154].

HP induced germination has been studied less extensively and in less depth in clostridia, but the picture that is emerging is that HP may induce germination in some, but not in all *Clostridium* species. A recent study revealed some interesting differences between *C. difficile* and *C. perfringens* [155]. *C. difficile* spores showed no germination at 150 MPa (37 °C) and did not lose their heat resistance, while *C. perfringens* spores became heat sensitive and released DPA, although they did not rehydrate completely. The authors proposed that the inability of *C. difficile* spores to germinate under mild HP was due to the absence of Ger-type receptors in this species. When the *C. perfringens* spores were heat activated before HP treatment, complete germination was demonstrated. HP treatments at 550 MPa (50 °C) led to DPA release in both clostridial species, although *C. difficile* remained heat resistant and did not complete germination. The 550 MPa treatment induced complete germination of *C. perfringens* spores only when they were first heat activated. This study opposes the results obtained in a previous study by Akhtar et al. (2009), who reported that HP does not trigger *C. perfringens* spore germination [156]. However, in this study no heat activation step was performed.

It has been shown that germination of *C. sporogenes* spores was only significantly induced by a mild HP treatment (200 MPa) when temperatures were above 40 °C and germinants were present [157]. Also gIICb spores do not seem to germinate under pressure at mild temperatures, although studies are very scarce. For *C. botulinum* type E spores, Reddy et al. (1999) reported that spores cannot be inactivated by high pressures (maximum tested: 827 MPa) at temperatures below 35 °C [158]. However, inactivation increases with increasing temperatures, resulting in ~3 log and ~5 log reduction of the type E strain Alaska spores at 40 °C and 50 °C respectively (827 MPa, 5 min). Unfortunately, germination was not documented in this work, although germination is assumed to be a crucial step in the HP-mediated inactivation

of spores at mild temperatures [158,159]. More recent work by Lenz et al. (2015) modeled HP thermal inactivation of type E spores, and also reported on spore germination [160]. The authors concluded that only very limited (maximum 0.6 log) germination was triggered by pressures up to 600 MPa, combined with temperatures up to 45 °C [160].

Scope of the work

Group II *C. botulinum* (gIICb) is an important pathogen of concern in mildly processed chilled ready-to-eat foods with an extended shelf life, because it can grow and produce the lethal botulinum neurotoxin during cooled storage. **Chapter 1** gave an overview of the current knowledge on this toxin and the lethal illness it causes, the heterogeneous species *C. botulinum*, and the hazards of gIICb in foods. **In Chapter 2,** the processes of sporulation and spore germination were described in more detail, because it is important to understand the properties of spores in order to understand the risk of gIICb in foods, and to develop adequate methods to control this risk, based on controlling spore germination and outgrowth.

In this PhD thesis, the general aim was to gain more insight in the germination mechanism of gIICb spores. Studies with these organisms are rather scarce in comparison with other foodborne pathogens, mainly because *C. botulinum* studies are subject to important biosafety and bioterrorism restrictions, and because their culture requires strict anoxic conditions. Moreover, gIICb are reported to be less amenable to genetic manipulation than other clostridia, and the genetic toolbox for these strains is still very limited. Therefore, we first aimed to construct nontoxigenic mutants by a novel knockout strategy that can provide a safe alternative for basic research with gIICb strains. Moreover, nontoxigenic mutants could be used as non-pathogenic surrogates that would greatly facilitate food challenge testing and process validation studies. There is a growing need for such studies in view of innovations in the food industry, that are driven by an increasing consumer's demand for fresh-tasting healthy ready-to-eat foods that have been minimally processed, contain less salt and no artificial preservatives, yet have a long shelf life. Furthermore, novel ingredients and novel food processing and preservation technologies have found their way to commercial food production, but data regarding their efficiency to control gIICb is lacking.

Since it is an advantage to have knowledge of the whole genome sequences (WGS), not only to support genetic analysis but also because they provide information on the metabolism and physiology, we assembled the WGS of three gIICb type E strains. In **Chapter 3** the WGS are presented of *C. botulinum* NCTC 8266 and NCTC 8550, and the nearly complete draft genome of NCTC 11219, consisting of four contigs. At the time these sequences were published, only two other WGS were available for gIICb type E strains: *C. botulinum* E1 strain Beluga (in six contigs) and strain Alaska E43.

In **Chapter 4** the construction of two nontoxigenic mutants of strain NCTC 11219 is reported. Besides the construction of an insertion mutant by the ClosTron system, we additionally developed a new gene replacement method that can be applied to delete any non-essential gene in gIICb and other clostridia. Moreover, the properties of both NCTC 11219 nontoxigenic mutants were compared with their parental strain, and this revealed some small changes in phenotype. Although it was concluded that the strains could still be useful as safe surrogates for challenge testing and basic research, we further investigated in **Chapter 5** if the observed changes were related to the toxin gene inactivation or whether they were the result of adventitious mutations that had occurred during mutant construction. This was done by constructing and analysing similar nontoxigenic mutants in a second gIICb strain (NCTC 8266), and by identifying all mutational changes of the mutants by WGS analysis.

Spore germination of *C. botulinum* NCTC 11219 Δ*bont* was studied in **Chapter 6**. First, different known germination inducers were tested on this particular strain and subsequently the inhibitory effect of four natural antimicrobial compounds (carvacrol, *trans*-cinnamaldehyde, carrot seed essential oil and hop β-acids) on germination was determined. Their activity has mostly been studied on vegetative bacteria, but only limited data are available on the effect of these natural compounds on endospores. Natural antimicrobials are being investigated to address concerns over the safety of some traditional preservatives like nitrite, and documenting the effect of these compounds on spore germination will contribute to a more complete assessment of their potential to control gIICb in foods.

In the following two chapters we attempted to enhance our understanding of the spore germination mechanism in gIICb by analysing the role of specific genes predicted to encode germination proteins. In **Chapter 7**, the inactivation of the only germinant receptor identified in gIICb strains (GerX3b) is decribed and its function in germination is examined. In **Chapter 8**, finally, we inactivated the cortex hydrolase SleB, and report on its role in spore germination.

CHAPTER 3*

Two complete and one draft genome sequence(s) of three nonproteolytic *Clostridium botulinum* type E strains NCTC 8266, NCTC 8550 and NCTC 11219

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* This chapter is based on the following publication: Clauwers, C., Briers, Y., Lavigne, R. & Michiels, C. Two complete and one draft genome sequence(s) of three nonproteolytic Clostridium botulinum type E strains NCTC 8266, NCTC 8550 and NCTC 11219. *Genome A.* **3**, e00083-15 (2015).

At the start of this PhD research, only two whole genome sequences were available for gIICb type E strains: *C. botulinum* E1 strain Beluga (six shotgun sequences; NZ_ACSC01000000) and strain Alaska E43 (whole genome; CP001078). Here, we present the high-quality whole genome sequences of *C. botulinum* type E NCTC 8266 and NCTC 8550 and the whole genome shotgun project of NCTC 11219, consisting of four contigs. Strains NCTC 8266 and 11219 are linked to botulism outbreaks caused by salmon (in 1944 and 1978) [161,162], NCTC 8550 has been isolated at the Pasteur Institute (Paris, France) in 1952.

To obtain high-quality genomic DNA (gDNA), formaldehyde was used for fixation of cells prior to isolation [163]. This step was essential to avoid DNA degradation by abundant extracellular DNase activity in gIICb cultures. Paired-end libraries were constructed using the NEBNext Ultra gDNA library prep protocol and analysed on the Agilent BioAnalyzer (VIB nucleomics core, Leuven, Belgium). Sequencing was performed on an Illumina Miseq sequencer, yielding 3,795,388; 3,669,738 and 4,733,175 reads of 150 bp for NCTC 8266, 8550 and 11219, respectively (with 157-, 152-, 187-fold total coverage). Genome assembly was performed using the CLC Genomics Workbench (v.7) (CLC Bio, Aarhus, Denmark) applying a combinatorial approach of reference assembly against *C. botulinum* Alaska E43 (3,659,644 bp), *de novo* assembly of non-assembled reads and manual editing.

Strains NCTC 8266 and 8550 are highly similar to strain Alaska (99 % identity) with a similar genome size of 3,611,897 and 3,611,898 bp. The draft genome sequence of NCTC 11219 (3,792,082 bp) differs more from Alaska (93 % identity). Gene predictions and annotations with the Prokaryotic Genome Annotation Pipeline identified 3218 genes for NCTC 8266 and 8550, and 3426 genes for NCTC 11219, of which 3060 and 3215 are protein-coding, respectively. For all three strains 34 rRNA and 79 tRNA sequences were found. PHAST (Phage Search Tool [164]) identified two potential prophages in NCTC 8266 and NCTC 8550, of which one appears intact (39.2 Kb) and one incomplete (24.4 Kb). In contrast, NCTC 11219 encodes five prophage regions, of which three potentially intact (39.6 Kb; 28.4 Kb; 26.1 Kb) and two incomplete (27.8 Kb; 13.9 Kb).

Nucleotide sequence accession numbers. The complete genome sequences have been deposited at DDBJ/ENA/GenBank under the accession no. CP010520 (NCTC 8266) and CP010521 (NCTC 8550). The whole genome shotgun project of NCTC 11219 has been deposited under the accession JXMR00000000. The version described in this paper is version JXMR01000000.

Genes of interest for this PhD work. The whole genome sequences of the three gIICb strains were searched by BLAST analysis for the presence of genes encoding BoNT using group II strain Alaska as a query sequence, and for genes with a possible role in spore germination as discussed in section 2.2 using query sequences from strain Alaska and group I strain ATCC 3502. In the following Chapters of this PhD, we specifically focused on *bont/E*, *gerX3b*, *sleB* and *sleC*.

Table 3.1: Overview of putative genes involved in BoNT production and spore germination that were (+) or were not (-) identified in the WGS of NCTC 11219, NCTC 8266 and NCTC 8550, using query sequences from group II strain Alaska and group I strain ATCC 3502. Gene content of strain Alaska is included for comparison.

	gIICb strains			
Homologous genes	**Alaska**	**NCTC 11219**	**NCTC 8266**	**NCTC 8550**
bont/E [a]	E3	E3	E1	E1
gerX3b (BAC)	+	+	+	+
sleB2	+	+	+	+
ypeB	-	-	-	-
cwlJ	-	-	-	-
sleC	+	+	+	+
sleC2a/b	b	b	b	b
sleM	+	+	+	+
csp	+	+	+	+
spoVA	CDE	CDE	CDE	CDE
sasp α/β	5	5	5	5
sasp γ	-	-	-	-
prkC	+	+	+	+

[a] The subtypes of the *bont* gene have been reported in literature [165].

CHAPTER 4*

Construction of nontoxigenic mutants of nonproteolytic *Clostridium botulinum* NCTC 11219 by insertional mutagenesis and gene replacement

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* This chapter is based on the following publication: Clauwers, C., Vanoirbeek, K., Delbrassinne, L. & Michiels, C. Construction of nontoxigenic mutants of nonproteolytic Clostridium botulinum NCTC 11219 by insertional mutagenesis and gene replacement. *Appl. Environ. Microbiol.* **82,** 3100-3108 (2016).

4.1 Introduction

Botulism is a rare but severe paralytic illness in humans and animals, caused by the botulinum neurotoxin (BoNT) produced by *C. botulinum*. Botulinum toxins are 150 kDa proteins with zinc endopeptidase activity, consisting of two subunits, a 100 kDa heavy chain and a 50 kDa light chain. The heavy chain is responsible for binding and translocation of the light chain into the cytosol of neuronal cells, whereas the light chain cleaves SNARE proteins that are involved in docking of acetylcholine-containing vesicles and fusion to the presynaptic membrane. When the SNARE proteins are cleaved, neurotransmitter release is inhibited, leading to paralysis of the corresponding muscle [166,167] (see 1.2).

C. botulinum is a strictly anaerobic bacterium that thrives in decaying organic matter in soils and sediments of ponds, lakes and oceans. It also forms dormant endospores that are highly resilient to hostile conditions and therefore can be found widespread in the environment. These spores may contaminate foods via the raw materials or other sources, possibly leading to foodborne botulism when they are not eliminated by processing and when their outgrowth is not controlled [76,168,169]. *C. botulinum* is divided into four distinct groups (I to IV) based on phylogenetic and physiological characteristics, of which only group I and II are typically associated with human botulism [49] (see 1.6). Group I *C. botulinum* consists of proteolytic mesophilic strains producing toxin types A, B and/or F. These strains form heat resistant spores and are the main target for the so-called "botulinum cook", the process used in canning of low acid foods (121 °C/3 min or equivalent). Group II *C. botulinum* (gIICb) comprises nonproteolytic strains producing toxin types B, E or F, which are saccharolytic, psychrotrophic (with minimum growth temperatures of 3 °C) and whose spores are less heat resistant than those of group I. In the food industry, gIICb is a major concern for the safety of refrigerated minimally processed foods of extended durability, because spores surviving the mild processing treatments may subsequently germinate, grow out and produce toxin during refrigerated storage [76,170,171]. The combination of the heat resistance of its spores and the ability to grow under refrigeration conditions makes this pathogen to be the main target pathogen that must be controlled in these foods. Guidelines for control of this hazard in the food industry have been developed more than two decades ago, requiring either a 6-D reduction of the spores by heat treatment (90 °C/10 min or equivalent), or control of outgrowth by limitation of the refrigerated storage time to < 10 days, inclusion of ≥ 3.5% NaCl, ≥ 100 ppm nitrite, acidifying to pH ≤ 5.0 or an appropriate combination of these or additional hurdles.

However, the increasing consumer's demand for fresh-tasting healthy ready-to-eat foods that have been minimally processed, contain less salt and no artificial preservatives, yet have a long shelf life, represent a challenge for the food industry in view of these botulinum safety guidelines. Furthermore, novel food processing and preservation technologies (e.g. high pressure or pulsed electric field treatment, natural preservatives) have found their way to commercial food production, but data regarding their efficiency to control gIICb is scarce [160,172–174] (see 1.7). This is in sharp contrast to the attention given in this context to other pathogens like *L. monocytogenes*, *Salmonella* and enterohemorrhagic *Escherichia coli*. The main reason for this paucity of data is that *C. botulinum* studies are subject to important biosafety and bioterrorism restrictions, and because their culture requires strict anoxic conditions. One possible approach to circumvent these difficulties is the use of nonpathogenic surrogate organisms. For example, *C. sporogenes* has been widely used as a surrogate for proteolytic *C. botulinum* in the studies on thermal processing of low-acid shelf-stable foods [175]. However, a suitable validated surrogate for gIICb is lacking up to date. Recently, Parker *et al.* (2015) studied previously isolated natural nontoxigenic *Clostridium* spp. that could possibly be used as surrogates for gIICb [176]. Two out of the three strains analysed showed equal or faster growth than toxigenic gIICb under most (but not all) stress conditions (low temperature, reduced pH and a_w), making them potentially useful for challenge studies with refrigerated foods, at least under some conditions. However, the spores of the three strains had lower heat resistance than some gIICb strains, and thus could not be used for validation of heating processes. Furthermore, it could not be excluded that the strains still carried *bont* or other toxin genes, and the (phylo)genetic relatedness of the strains to gIICb was not documented. This makes the behaviour of these strains under other than the tested environmental conditions unpredictable (e.g. sensitivity to nitrite and other preservatives, sensitivity to organic acids…).

As an alternative approach to develop safe strains for challenge studies and process validation, we attempted in the present work to construct nontoxic derivatives from a toxic gIICb strain by making a targeted knockout of the *bont* gene. The resulting derivative strains are then expected to differ from their parent only in toxin production, and thus to be a highly reliable alternative for challenge studies. Although the inactivation of specific genes in clostridial species has proven to be a rather difficult, slow and inefficient task for a long time, the genetic toolbox for knockout mutagenesis has been expanding the last few years. The ClosTron system, which makes use of a mobile intron that can be retargeted to a sequence of interest, has proven to be particularly efficient for insertional mutagenesis in a range of clostridial species [177], but only a few studies have used ClosTron mutagenesis in gIICb thus far [178,179].

Interestingly, ClosTron has been used to knockout the *bont* gene both in gICb and gIICb [178,180], but the properties of the gIICb knockout strain in view of its possible usefulness for challenge or process validation studies in foods were not further investigated. Importantly, a disadvantage of ClosTron mutagenesis is that reversion to the toxigenic state by excision of the intron from the *bont* gene cannot be excluded. For this reason, we additionally developed a method for *bont* deletion that makes use of *pyrE*, a gene encoding the enzyme orotate phosphoribosyltransferase which is required for *de novo* pyrimidine biosynthesis. This gene has been used before in various clostridia (*C. sporogenes*, *C. difficile*, *C. acetobutylicum*) as a positive/negative selection marker. PyrE is essential for growth in the absence of uracil, while cells lacking PyrE become resistant to 5-fluoroorotic acid (5-FOA), a substrate analogue in *de novo* uracil biosynthesis that is converted by PyrE to a toxic metabolite. Therefore, cells encoding *pyrE* can be selected on uracil-free medium or counterselected in the presence of 5-FOA [181–183]. We used this bidirectional selection to delete *bont/E* and replace it by an erythromycin resistance cassette. This is the first report of a targeted gene deletion in gIICb. In addition, our gene replacement strategy is novel in that it allows a single-step selection of gene replacement by double homologous recombination and loss of the plasmid on which the donor DNA is supplied.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Cultures of *C. botulinum* type E NCTC 11219 (obtained from National Collection of Type Cultures, Public Health England) were routinely grown at 30 °C in trypticase peptone glucose yeast extract broth (TPGY; 50 g/l trypticase (Becton-Dickinson, MD, USA), 5 g/l bacteriological peptone (Oxoid, Basingstoke, UK), 20 g/l yeast extract (Oxoid), 4 g/l glucose (Acros, New Jersey, USA), 1 g/l sodium thioglycollate (Sigma, Steinheim, Germany)) and plated on reinforced clostridial medium (RCM (Thermo Fisher Diagnostics, Groot-Bijgaarden, Belgium); 37 g/l RCM + 15 g/l agar), TPGY agar (TPGY broth + 15 g/l agar) or tryptone yeast extract thioglycollate agar (TYG; 30 g/l tryptone (Lab M, Heywood, UK), 20 g/l yeast extract, 1 g/l sodium thioglycollate, 15 g/l agar). Trypticase glucose yeast broth (Bio-Rad, CA, USA) supplemented with 0.1 % trypsin (Thermo Scientific, Darmstadt, Germany) was used to grow cultures for the mouse bioassays. Uracil-deficient medium was accomplished by replacing yeast extract in TPGY agar with 20 g/l Acid Hydrolysed Casein (Lab M)[184]. Clostridial vegetative cultures were manipulated and incubated in a Don Whitley

DG250 anaerobic workstation (initial gas mixture comprised of 80% N_2, 10% CO_2, and 10% H_2) using overnight pre-reduced media. *E. coli* strains were grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) or on LB agar (LB + 15 g/l agar) at 37 °C. *E. coli* DH5α was used for cloning and maintenance of plasmids, while *E. coli* CA434 (HB101 containing plasmid R702, [185]) was used as conjugation donor. Media were supplemented with the following antibiotics (Applichem, Darmstadt, Germany): thiamphenicol (Tm, 15 µg/ml in agar, 7.5 µg/ml in broth) and erythromycin (Em, 2.5 µg/ml) for *C. botulinum*, and cycloserine (Cy, 250 µg/ml) and chloramphenicol (Cm, 25 µg/ml in agar, 12.5 µg/ml in broth) for *E. coli*. 5-Fluoroorotic acid (5-FOA, 500 µg/ml (Manchester Organics, Chesire, UK)) was used for the screening of uracil auxotrophs.

4.2.2 Sporulation and purification of spore crops

Spore crops were prepared using a two-phase sporulation medium as described before, with minor adjustments [186]. First, a single colony was inoculated in 1 ml TPGY broth at 30 °C. After 24 h of growth, this culture was added to a two-phase medium consisting of 4 ml distilled deoxygenated water over solid sporulation medium (3 g cooked meat medium (Oxoid), 0.03 g glucose, 0.45 g agar in 30 ml water). Spore production in this medium was regularly monitored by phase-contrast microscopy (DMLB Leica, Diegem, Belgium), by placing 2 µl of the culture on a microscopy slide, and observing the presence of phase-bright or phase-dark spores.

Spores were harvested from the liquid phase after six days incubation at 30 °C by centrifugation (3400 × *g*, 4 °C, 15 min). The resulting pellet was washed four times with 0.85 % sterile saline by centrifugation, concentrated fivefold and stored in saline at 1-4 °C outside the anaerobic workstation.

4.2.3 Construction of plasmids

The ClosTron technology was used for the generation of an insertion mutant. This system is based on retargeting a bacterial group II intron to insert in a gene of interest [177,187]. Possible target sites in the *bont/E* gene of *C. botulinum* NCTC 11219 were identified using an intron design tool on the ClosTron website (www.clostron.com), and one site was chosen based on a high score according to the algorithm as well as proximity to the N-terminus of the toxin. The plasmid pMTL007C-E2:Cbo:*bontE*-211a, containing the intron flanked by the specific targeting sequences was obtained from DNA 2.0 Inc. (Menlo Park, CA, USA).

(GGTCATAATAACTACTATCTCCATTTTTTA<intron>CTAATTATTTACAAA)

The second knockout strategy consisted of an allelic exchange of *bont/E* with *ermB*, an Em resistance gene. A novel three-step strategy was designed, with each step requiring a specific plasmid construct. Oligonucleotide primers used in these constructions are listed in Table 4.1 and were obtained from Integrated DNA Technologies (Heverlee, Belgium). Constructs were routinely transferred to *E. coli* DH5α by electroporation and verified by sequencing before further use. The first plasmid construct was used to generate a *pyrE* deletion (locus tag SR42_16845) in strain NTCT 11219. To this end, flanking loci of *pyrE* were cloned in pMTL84151 [188] to yield pMTL84151_*pyr5'pyr3'*. More specifically, the 5' fragment (1086 bp) was amplified from gDNA of NCTC 11219 with primers pyrE_5'F/pyrE_5'R and the 3' fragment (1164 bp) with primers pyrE_3'F/pyrE_3'R. Amplicons were restricted with respectively KpnI/BamHI, and BamHI/XhoI, ligated end-to-end in pMTL84151 opened with KpnI and XhoI, and electroporated to *E. coli* DH5α.

Secondly, the plasmid pMTL84151_Δ*bont*, used to replace *bont/E* for *ermB* was constructed as illustrated in Fig. 4.1. Flanking loci (5' locus: 1080 bp; 3' locus: 1261 bp) of *bont/E* were first cloned in pMTL84151, using primers bontE_5'F/bontE_5'R and bontE_3'F/bontE_3'R for amplification. These loci include the first two codons of *bont/*E in the 5' fragment and the last sixteen codons in the 3' fragment. After restriction of the PCR products with respectively KpnI/BamHI and BamHI/XhoI, the 5' and 3' fragments were ligated end-to-end in pMTL84151 opened with KpnI and XhoI. Subsequently, *ermB* (amplified from pMTL82254 with pMTL82254ermB_F/pMTL82254ermB_R and restricted at both ends with BamHI) was inserted in the BamHI site of this plasmid, resulting in pMTL84151_*5'bont_ermB_3'bont*. In addition, wild type *pyrE* of NCTC 11219 (675 bp) was amplified from start to stop codon with primers pyrE11219_F/pyrE11219_R, restricted with NdeI/SacI and placed after promotor p_{fdx} in pMTL83353 opened with the same enzymes. p_{fdx} was used to drive *pyrE* expression because *pyrE* is located at the end of an operon in NCTC 11219 and thus could not be simply transcribed with its native promotor. Hereafter, the fragment containing p_{fdx} and *pyrE* was amplified with pMTL83353_F/pyrE11219_R, digested with SbfI and SacI and cloned in pMTL84151_*5'bont_erm_3'bont*, restricted with the same enzymes. This resulted in the plasmid pMTL84151_Δ*bont*.

A third plasmid was designated pMTL84151_WT*pyr*, used for restoring the *pyrE* deletion back to wild type. This was created by cloning a *pyrE* amplicon with its 5' and 3' flanking regions (2956 bp) generated with primers pyrE_5'F/pyrE_3'R and restricted with KpnI and XhoI, into pMTL84151, cleaved with the same enzymes. In this construct, a promotor is absent thus *pyrE* is not expressed on the plasmid.

Table 4.1: PCR oligonucleotides used for cloning and construct verification. Restriction sites are underlined: KpnI (GGTACC), BamHI (GGATCC), XhoI (CTCGAG), NdeI (CATATG), SacI (GAGCTC) and SbfI (CCTGCAGG). The primers' coordinates of NCTC 11219 are indicated in the third column, based on accession numbers JXMR01000001[a] or JXMR01000004[b].

Name	Sequence (5'-3')	Coordinate of 3' end
ClosTron		
bontE_F	CCAGGCGGTTGTCAAGAATTTTAT	2298420[a]
bontE_R	TCAAATAAATCAGGCTCTGCTCCC	2298057[a]
RAM_F	ACGCGTTATATTGATAAAAATAATAATAGTGGG	
RAM_R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG	
pCD6_F	GTTGGGAGTAGTTGTGC	
pCD6_R	ATGGTATCTCATTATTGGC	
ClosTron_R	GTTTCAGACACTTTCCTCTATCGAG	
Y-linker primer	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG	
ΔpyrE		
pyrE_5'F	AACGGTACCCCACTACGTTCTCTCTTAGAGG	246521[b]
pyrE_5'R	AACGGATCCTGAGTTTAAGGTTTTTAGTTGG	247564[b]
pyrE_3'F	CATGGATCCTCCTCCCAAATTTCATAATGT	248312[b]
pyrE_3'R	CATCTCGAGTGTTTTATGCTGTGGTCCTG	249436[b]
pyrE_5'Fb	CCAACTTTATCAAAAGCTCAG	246369[b]
pyrE_3'Rb	CTAATACAGGAAAGCATGGC	249503[b]
pMTL84151_mcsF	AGGAAACAGCTATGACCG	
pMTL84151_mcsR	GACGTTGTAAAACGACGG	
ΔbontE		
pyrE11219_F	AGGCATATGGAAGCATATAAAAAAGAG	248264[b]
pyrE11219_R	CTTGAGCTCCTACTTAGCACCATATTC	247627[b]
bontE_5'F	TAAGGTACCGTTATTGGAGATACATCCGG	2299564[a]
bontE_5'R	TAAGGATCCTGGCATATACAGCATCTCC	2298522[a]
bontE_3'F	TAAGGATCCAGAGATCATACAAACAGCAATGG	2294800[a]
bontE_3'R	TAACTCGAGCTATTCGAGAGAGCATTGGTCG	2293583[a]
upbontE_F	GAACTACTTATAAAAGAACAAACCTCACC	2299627[a]
downbontE_R	GAATGGATATCTTAGGATAATCATTCCAC	2293466[a]
pMTL83353_F	GAGCCTGCAGGATAAAAAAATTGTAG	
pMTL82254ermB_F	GTTGGATCCGAAGCAAACTTAAGAGTGTG	
pMTL82254ermB_R	TACGGATCCACATTCCCTTTAGTAACGTG	

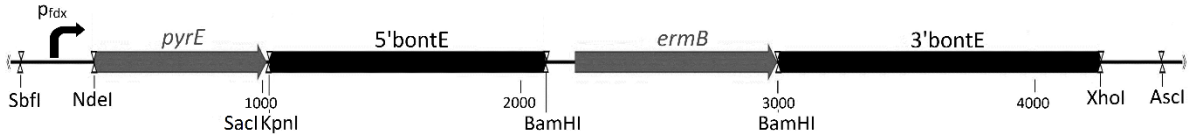

Fig. 4.1: Scheme showing the different fragments cloned between SbfI and AscI restriction sites of shuttle vector pMTL84151 (Tm^R) to create pMTL84151_Δ*bont* (Tm^R Em^R), that was used for replacing the *bont/E* gene for an *ermB* marker. The p_{fdx} promotor (bended arrow) was derived from pMTL83353 and drives expression of *pyrE*, which was amplified from gDNA of NCTC 11219. The 5' and 3' fragments of *bont/E* were also amplified from NCTC 11219 gDNA, and flank *ermB* derived from pMTL82254.

4.2.4 Mating

Each of the four plasmid constructs described above was introduced in *E. coli* CA434 by electroporation and selecting Cm^R transformants (the *catP* marker encodes Cm/Tm resistance; Cm is used in *E. coli* whereas Tm is used in *C. botulinum*). Hereafter, plasmid transfer to *C. botulinum* was accomplished by conjugation. The pMTL84151 backbone carries the Gram negative ColE1 replicon (theta replication), the transfer functions of incP plasmid RK2, and the Gram positive pCD6 replicon of which the exact mechanism of replication is not clear [189,190].

For plasmid transfer to *C. botulinum* NCTC 11219, one ml of an overnight LB culture of *E. coli* donor containing the appropriate plasmid was centrifuged, the pellet was washed with PBS (phosphate buffered saline, 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH adjusted with HCl to 7.4) and taken in the anaerobic workstation. Next, the pellet was resuspended in 200 µL of the NCTC 11219 recipient grown for 24 h in TPGY broth, whereafter the mixture was spread on a cellulose acetate filter (poresize 0.45 µm) on non-selective TYG agar. After 24 h of incubation at 30 °C, the cells were washed off the filter with 1 mL PBS, and 200 µL was plated on selective RCM plates (Tm for resistance encoded on the plasmid, Cy against *E. coli* donor). Colonies appearing within three days were restreaked to purity on the same selective medium.

4.2.5 Isolation and verification of *bont/E* ClosTron insertion mutant

To confirm transfer of the ClosTron plasmid pMTL007C-E2:Cbo:*bontE*-211a to NCTC 11219, Tm^R colonies were purified after mating and analysed by PCR using primers RAM_F/RAM_R. Subsequently, transconjugants were streaked on RCM supplemented with Em, and well-developed colonies appearing within three days were restreaked on the same medium for purification. PCR and sequence analysis with primers bontE_F/bontE_R were performed to verify successful intron insertion in *bont/E*. Individual colonies from a verified

clone were then screened for Tm sensitivity, indicating loss of the ClosTron plasmid, which was hereafter confirmed by PCR using primers pCD6_F/pCD6_R.

To investigate whether only one insertion event took place, the Y-linker PCR described by Kwon & Ricke was used [191]. In this method, gDNA of the ClosTron mutant is restricted to completion with NlaIII and ligated to a Y-shaped linker, generated by mixing two oligonucleotides that are complementary only at one end. The complementary parts of these oligonucleotides form the stalk of the linker, which has a 3' CATG overhang that stocks to the NlaIII chromosomal DNA ends. The non-complementary parts of the oligonucleotides form the Y-linker arms. PCR is then conducted on the ligation mixture with a ClosTron specific primer (ClosTron_R) and a Y-linker primer with a sequence identical, not complementary, to one of the arms. Only when the ClosTron_R primer extends DNA synthesis during the first PCR cycle into the Y-linker arm, the Y-linker primer can anneal and a PCR product will be formed. Consequently, the two primers selectively amplify all fragments in the genome containing the ClosTron insert so that the amount of insertions could be determined.

4.2.6 Isolation and verification of *bont/E* deletion mutant

After conjugation of pMTL84151-*pyr5'pyr3'* to NCTC 11219, transconjugants were streaked on RCM supplemented with 5-FOA to select for PyrE deficient mutants generated by double homologous recombination between the plasmid-based *pyrE*-flanking fragments and the corresponding chromosomal sequences. Deletion was confirmed by PCR and sequence analysis with primers pyrE_5'Fb/pyrE_3'Rb, which anneal outside the homologous fragments involved in recombination. Further, a clone from which the plasmid was cured, was isolated by screening for Tm sensitivity. Next, NCTC 11219 Δ*pyrE* was mated with *E. coli* CA434 containing pMTL84151_Δ*bont*. Transconjugants were streaked on RCM supplemented with Em and 5-FOA, to select for clones in which double homologous recombination as well as loss of the plasmid had occurred. Plasmid loss was confirmed by the Tm sensitivity of the clones. PCR and sequence analysis with primers upbontE_F/downbontE_R, annealing outside the homologous fragments, were performed to confirm that *bont/E* was deleted and replaced by *ermB*. Finally, the *pyrE* gene was restored to wild type by first conjugating pMTL84151-WT*pyr* to NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB* and subsequently propagating one transconjugant in liquid TPGY medium for about 60 generations and subsequently plating on uracil-deficient medium. Only clones in which the chromosomal *pyrE* gene is restored by allelic exchange with the plasmid-based promotorless *pyrE* grow well on these plates. One such clone, confirmed by PCR and sequence analysis to

be identical to the wild type in the *pyrE* locus and from which the plasmid was cured as explained before, was isolated and designated NCTC 11219 Δ*bontE*::*ermB*.

4.2.7 Mouse bioassays

Mouse bioassays were performed for detection of active botulinum toxin in clostridial cultures. The assays were performed with *bont/E* knockout strains NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB*, and with the corresponding positive control strains NCTC 11219 wild type and NCTC 11219 Δ*pyrE*, respectively. Supernatants from five-day cultures of the strains in trypticase glucose yeast broth supplemented with trypsin at 30 °C were used for toxin testing. A seroneutralization test using anti-E antitoxin which specifically neutralizes the toxic effects of BoNT/E was included. Neutralized (i.e. mixed with antitoxin) and untreated filtered supernatants were injected intraperitoneally in mice for evaluating the toxicity. Two animals were used per sample. Mice were observed at regular intervals for a period of four days for signs of botulism (ruffling of the fur, hypotonic abdomen, wasp-waist, difficulty in breathing, weakness of the limbs, and total paralysis) or death. Typical paralysis and/or death of the mice with prevention of these effects by the administration of antitoxin establishes a positive test for BoNT/E.

4.2.8 Phenotypical analysis of wild type NCTC 11219 and NCTC 11219 *bont/E* mutants

Unstressed growth. Wild type *C. botulinum* NCTC 11219 and mutants *bontE*211a::CT and Δ*bontE*::*ermB* were inoculated in triplicate (three independent single colonies of each strain) in 1 ml TPGY broth and incubated for 24 h at 30 °C. The stationary cultures were diluted $5x10^3$-fold in 50 mL TPGY (= t_0), and incubated at 30 °C. Cell numbers were determined by plate counting on TPGY agar every 2 h during 16 h and again 8 h later.

Stressed growth. Six independent overnight cultures of wild type and mutants were streaked on RCM plates with different pH (5.7, 5.5, 5.2, 4.9; adjusted with HCl and measured before and after autoclaving) or NaCl content (1.9 %, 2.1 %, 2.3 %, 2.5 %; taking into account that 0.5 % NaCl is already present in RCM). The plates were incubated at 30 °C in an anaerobic workstation and colony formation was observed macroscopically for several days. Growth at lower temperatures was analysed by streaking six independent single colonies from each strain that were pregrown at 12 °C on RCM plates, and incubating the plates in AnaeroGen bags (Oxoid) at 8 °C and 12 °C. Colony formation was observed daily, and the time that was required to form 1 mm colonies was noted for each replicate.

Heat resistance of spores. Spore crops were made in triplicate from different colonies per strain and used within two weeks for this experiment. The initial spore count was determined

by plate counting on TPGY after a heat treatment to inactivate remaining vegetative cells (65 °C, 10 min), but non-preheated samples were used for the heat inactivation experiment. Heat treatments were conducted in a heating block at 70 °C, 73 °C, 90 °C and 93 °C. At different time points, samples were taken and cooled immediately to stop inactivation, diluted in 0.85 % NaCl, plated on TPGY and incubated at 30 °C for colony counting. For the treatments at 90 °C and 93 °C, lysozyme (10 µg/ml; Carl Roth GmbH, Karlsruhe, Germany) was added to the plating medium. The D-value was determined as the negative reciprocal of a regression line fitted on a plot of the logarithm of the plate count versus time.

Onset of sporulation. Single colonies were inoculated in 1 ml TPGY broth in triplicate and incubated for 14 h at 30 °C. Then, these cultures were diluted hundredfold in 5 mL TPGY (= t_0), and incubated at 30 °C. After different incubation times, aliquots were heat treated (65 °C, 10 min) in a heating block and then plated on TPGY plates, to determine the spore counts. Total cell numbers were determined by plate counting without receiving the heat treatment.

4.2.9 Statistical analysis

To statistically assess the equivalence of unstressed growth, spore yield, spore fraction and spore heat resistance between the wild type and the *bont/E* mutants, the two-tailed unpaired Student's t test was used with a significance level of 0.05. Since growth at low temperature was analysed in a 'time to colony formation' experiment, in this case a logistic regression model was fitted using the day, temperature and strain as predictor variables. The proposed model was highly significant (P-value whole model likelihood ratio test < 0.001; generalized $R^2 = 0.9063$) and showed no lack of fit (P-value lack of fit likelihood ratio test ~ 1), indicating it was suitable for testing the individual parameters.

4.3 Results and Discussion

C. botulinum NCTC 11219 is a group II type E strain, of which we reported the genome sequence in four contigs (accession number JXMR01000001-JXMR01000004) in Chapter 3 [192]. Here, we used this strain for the construction of atoxigenic mutants. The ClosTron system was used for insertional inactivation of the *bont/E* gene, whereas a new approach based on double homologous recombination using the selection markers *pyrE* and *ermB* was designed to create a *bont/E* deletion.

4.3.1 Construction of insertion mutant *C. botulinum* NCTC 11219 *bontE*211a::CT

Plasmid pMTL007C-E2:Cbo:*bontE*-211a, containing an intron retargeted to insert in *bont/E*, was transferred by conjugation to NCTC 11219. Since the intron contains a retrotransposition-activated marker (RAM) based on the *ermB* gene, successful insertion of the intron in the genome is selectable by the expression of Em resistance. Em^R clones were picked up and analysed by PCR and sequence analysis. The intron was correctly located in one clone at position 211 in the *bont/E* open reading frame with an antisense orientation of *ermB* relative to *bont/E* (Fig. 4.2, Fig. 4.3 lane 2-3). Further analysing this clone by Y-linker PCR and sequencing of the PCR product, confirmed that only a single intron was present in the genome. The efficiency of the different steps in the ClosTron mutagenesis procedure was rather low. For conjugation of the ClosTron plasmid to NCTC 11219, several attempts were needed to obtain only two transconjugants. Similarly, several clones of transconjugants had to be streaked to obtain fifteen Em^R colonies, and only one of these had the correct intron insertion. This confirms earlier statements in the literature that *C. botulinum* is less genetically tractable than most other clostridia [178,193].

4.3.2 Construction of deletion mutant *C. botulinum* NCTC 11219 Δ*bontE*::*ermB*

A deletion strategy to knockout *bont/E* was developed by combining different genetic tools that have not yet been used in gIICb, despite their success in other clostridia. Since effective suicide plasmids have not yet been described for *C. botulinum*, and given the reported low frequency of DNA transfer into gIICb, we chose to make use of pseudo-suicide plasmids for conjugation [187,194]. These plasmids autonomously replicate but are segregationally unstable, providing the advantage to be lost more rapidly in the absence of selection after successful conjugation. A set of modular shuttle vectors, the pMTL80000 series, carrying four different Gram-positive replicons was constructed by Heap *et al.* (2009) for use in the clostridia [188]. We selected pMTL84151 with the pCD6 replicon from this set since this plasmid has the lowest stability in gICb and gIICb ([188], unpublished results). Another key element in the knockout

strategy was the use of the *pyrE* gene as a bidirectional selection marker. First, *pyrE* was deleted from NCTC 11219 resulting in a 5-FOA resistant and uracil auxotroph strain (Fig. 4.3. Lane 4-5). Then the obtained Δ*pyrE* mutant was used as background to delete *bont/E* and replace it with an *ermB* marker. This was achieved by first conjugating pMTL84151_Δ*bont* (Fig. 4.1) to the Δ*pyrE* mutant and selecting for Tm resistance, then streaking a transconjugant on medium containing both Em and 5-FOA. Since the plasmid expresses *pyrE* *in trans*, 5-FOA selects for its loss. Further, clones that lost the plasmid can only remain resistant to Em when the *ermB* gene was exchanged with the *bont/E* gene by double homologous recombination. Although this procedure selects for two events simultaneously, the frequency was sufficient to allow isolation of several colonies from a single streak. Obviously, the efficiency benefits from the low segregational stability of the plasmid. Loss of the plasmid was confirmed by loss of Tm resistance, and PCR and sequence analysis confirmed the replacement of *bont/E* by *ermB* (Fig. 4.2, Fig. 4.3 lane 6-7).

Finally, the *pyrE* deletion was restored to wild type by swapping *pyrE* and its flanking regions back to the chromosome by double homologous recombination with pMTL84151_WT*pyr*. To this end, the plasmid was first conjugated to NCTC 11219 Δ*pyrE* Δ*bontE*::*ermB*. The Tm^R transconjugants remained resistant to 5-FOA, which confirmed that *pyrE* was not expressed on pMTL84151_WT*pyr*. Since *pyrE* was cloned without its promotor (which is further upstream the *pyr* operon) on this plasmid, this result indicates that there is no leaky expression from plasmid promoters. In line with this observation, the transconjugants did not grow when they were plated on uracil-deficient medium, except for some rare colonies which showed normal growth when restreaked on the same medium. From these, subclones having lost the plasmid with a Tm^S phenotype were isolated. These were confirmed to have a wild type chromosomal *pyrE* allele, and were designated as NCTC 11219 Δ*bontE*::*ermB*.

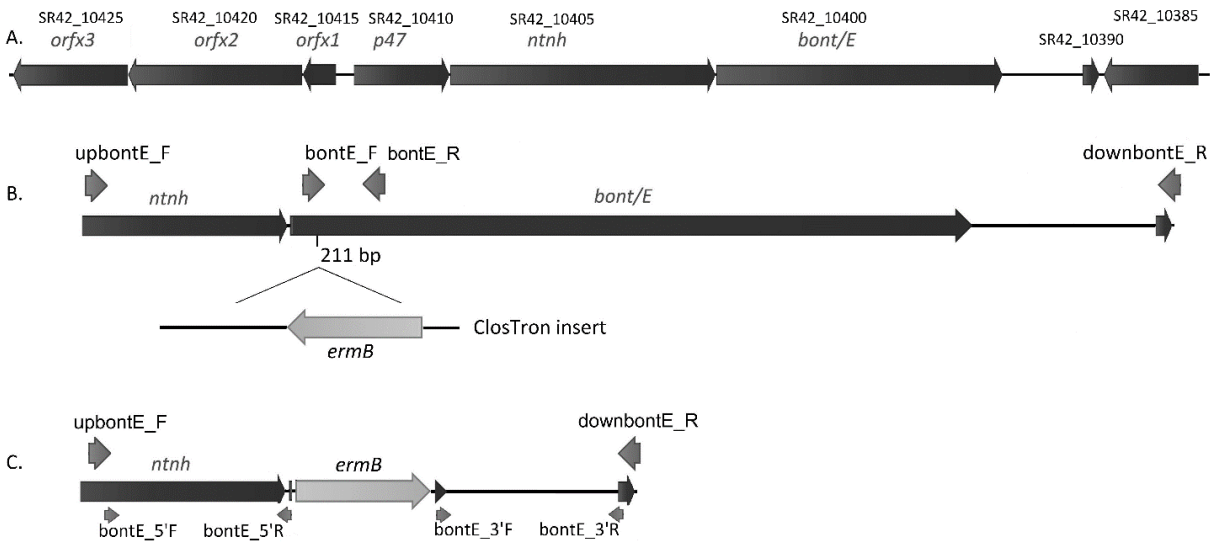

Fig. 4.2: Schematic representation of A) *bont/E* with its upstream and downstream genes in *C. botulinum* NCTC 11219. Locus tags for all genes are also shown. B) Position of the ClosTron insert after the 211 th nucleotide of the *bont/E* open reading frame in the NCTC 11219 *bontE*211a::CT mutant, with *ermB* positioned antisense to *bont/E*. Also shown are primers bontE_F/bontE_R, annealing up- and downstream of the ClosTron target site, and primers upbontE_F/downbontE_R, annealing outside the loci used in double homologous recombination to delete *bont/E*. C) NCTC 11219 Δ*bontE*::*ermB* mutant with *bont/E* deleted and replaced by *ermB*, confirmed by PCR using primers upbontE_F/downbontE_R. The positions of the cloning primers bontE_5'F/bontE_5'R and bontE_3'F/bontE_3'R, used for construction of pMTL84151_Δ*bont*, also are indicated.

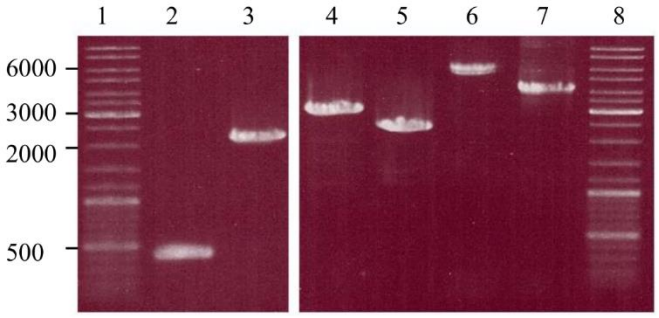

Fig. 4.3: PCR analysis of different strain constructs. Lane 1 and 8: Molecular size marker (GeneRuler from Thermo Scientific); lane 2: NCTC 11219, *bont/E* fragment amplified with primers bontE_F/bontE_R (expected size: 410 bp); lane 3: NCTC 11219 *bontE*211a::CT, *bont/E* fragment with ClosTron insertion amplified with primers bontE_F/bontE_R (expected size: 2008 bp); lane 4: NCTC 11219, *pyrE* region amplified with primers pyrE_5Fb/pyrE_3Rb (expected size: 3174 bp); lane 5: NCTC 11219 Δ*pyrE*, *pyrE* region amplified with primers pyrE_5Fb/pyrE_3Rb (expected size: 2474 bp); lane 6: NCTC 11219 Δ*pyrE*, *bont/E* region amplified with primers upbontE_F/downbontE_R (expected size: 6218 bp); lane 7: NCTC 11219 Δ*bontE*::*ermB*, *bont/E* region amplified with primers upbontE_F/downbontE_R (expected size: 3449 bp).

4.3.3 Mouse bioassays

The presence of BoNT/E in culture supernatants of the NCTC 11219 wild type and the Δ*pyrE* strain was confirmed whereas no active toxin was detected for the *bont/E* insertion and deletion mutants. All mice injected with supernatant of wild type and Δ*pyrE* died within a day, while mice injected with supernatants of the mutants or supernatants from the *bont/E* wild type strains mixed with antitoxin E, survived till the end of observation at four days. Thus, loss of toxicity in both mutants was confirmed. In addition, the seroneutralization test using anti-E antitoxin confirmed that only one neurotoxin is produced by NCTC 11219, as was already predicted from the genome sequence (Chapter 3,[192]).

4.3.4 Phenotypical analysis of wild type NCTC 11219 and NCTC 11219 *bont/E* mutants

Nontoxic mutants of gIICb have the potential to greatly facilitate basic research and food challenge studies with this organism, provided that their properties related to growth, sporulation, survival and inactivation are not modified due to the knockout of the *bont/E* gene. Here, we compared the growth, sporulation and spore heat resistance of both constructed *bont/E* mutants and the wild type NCTC 11219 strain.

Unstressed growth. The growth curves of the mutants and the wild type at 30 °C were very similar (Fig. 4.4). The DMFit software was used to determine the maximum growth rate μ_{max} and the upper asymptote y_{end}. The lag phase could not be evaluated because there were not enough points available to measure this accurately. No significant differences existed between μ_{max} and y_{end} of the wild type, ClosTron and Δ*bontE* mutant. The values were 0.7 +/- 0.2, 0.5 +/- 0.1, 0.4 +/- 0.0 (log CFU/ml)/h for μ_{max}, and 7.8 +/- 0.3, 8.0 +/- 0.0; 8.0 +/- 0.1 log CFU/ml for y_{end}, respectively.

Stressed growth. A quantitative comparison of growth under acidic, NaCl and low temperature stress was performed by assessing the time to colony formation on RCM plates using six replicates per strain. On acidified RCM, results of both mutants and wild type were identical in sixfold. Single colonies of 1 mm were visible after 24 h at pH 5.7 and after 30 h at pH 5.5, while pinpoint colonies appeared after 72 h at pH 5.2. No colonies were formed at pH 4.9 up to 5 days. Under NaCl stress, all three strains showed clear single colonies in sixfold after 24 h at 1.9 % and 2.1 % NaCl. Unexpectedly, a small difference was observed at 2.3 % NaCl between the Δ*bontE*::*ermB* strain compared to the wild type and ClosTron mutant. Strain Δ*bontE*::*ermB* formed clear 1 mm colonies after 24 h (sixfold) while those of the wild type and ClosTron mutant were pinpoint sized, and reached 1 mm only after 48 h (sixfold). No colonies appeared on 2.5 % NaCl for any strain. The third stress condition tested, was growth at low

temperature. Single colonies grown at 12 °C on RCM were restreaked in sixfold on RCM, then incubated at 12 °C and 8 °C, and the time to formation of colonies (1 mm) was registered. As opposed to the data for acid and NaCl stress, the time to colony formation at low temperature showed variability among the six replicates per strain as well as between strains, as indicated in Table 4.2. A logistic regression model was fit on this data and showed that there was no significant effect of the strain (P-value effect likelihood ratio test 0.2388). Therefore, it can be concluded that there is no significant difference in growth at low temperature between the three strains.

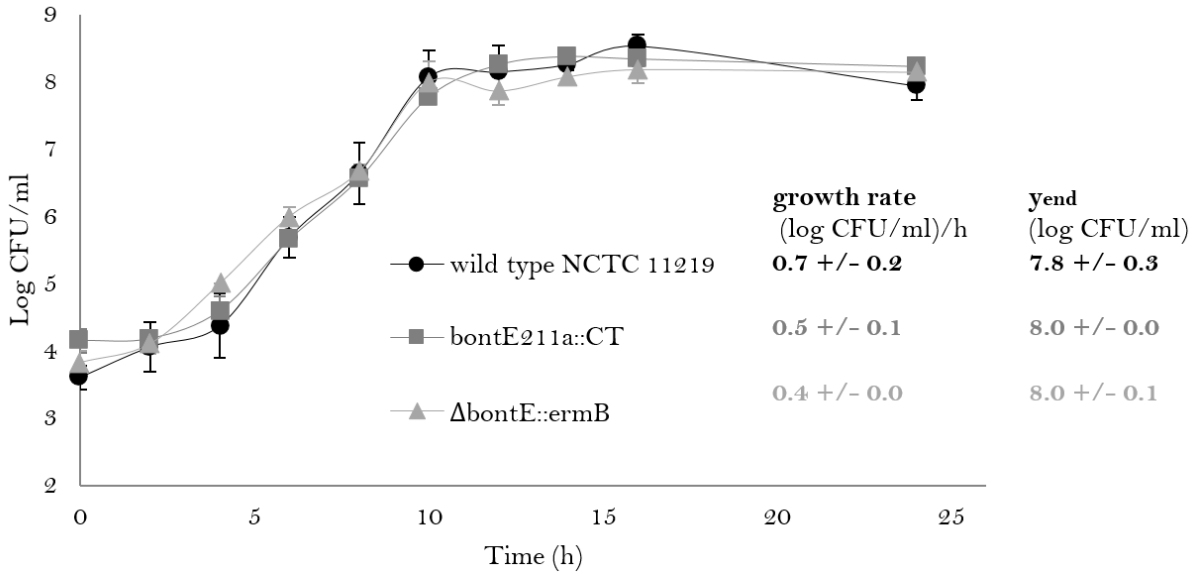

Fig. 4.4: Growth in TPGY broth at 30 °C of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* determined by plate counting on TPGY. Means +/- standard deviations are shown from three independent replicates of each strain. The growth curves were modeled with DMFit to determine the growth rate and upper asymptote y_{end}. No significant differences were found between the three strains ($p > 0.05$).

Table 4.2: Growth at 12 °C and 8 °C of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* on RCM plates. Single colonies were restreaked in sixfold and the time to formation of 1 mm colonies was noted.

	Number of plates showing single colonies				
	at 12 °C		**at 8 °C**		
Days (d)	**3 d**	**4 d**	**7 d**	**8 d**	**9 d**
NCTC 11219	5/6	6/6	4/6	5/6	6/6
*bontE*211a::CT	4/6	6/6	4/6	6/6	6/6
Δ*bontE*::*ermB*	3/6	6/6	3/6	4/6	6/6

Spore production. Sporulation was performed in two-phase sporulation medium and observed regularly with phase-contrast microscopy. Spore formation started within 72 h for all strains. Spore counts were determined after six days by plate counting after inactivation of

the vegetative cells at 65 °C for 10 min. No differences were observed in spore yield between wild type, *bontE*211a::CT and Δ*bontE*::*ermB*. Spore crops used for treatments at 90 °C/93 °C had a yield of 7.8 log +/- 0.1 CFU/ml, 8.1 +/- 0.3 CFU/ml and 7.9 +/- 0.1 CFU/ml, respectively. The spore crops used for heat treatments at 70 °C/73 °C all had the same yield, 7.6 log +/- 0.1 CFU/ml.

The NCTC 11219 ClosTron mutant shows an early onset of sporulation. Although the spore yield after six days was identical for the NCTC 11219 mutants in comparison with the other strains, we observed with microscopy that particularly high numbers of NCTC 11219 *bontE*211a::CT spores were already present in early stationary-phase cultures while this was not the case for the deletion mutant (note: the wild type strain could not be observed under the microscope because of biosafety restrictions). To compare the degree of sporulation in the early stationary phase, we determined the spore counts as well as the total counts in cultures of the ClosTron mutant and the wild type strain at different time points. It is important to note that sporulation is assessed here in TPGY growth medium, not in the sporulation medium that is used for sporulation (which follows the six days protocol).

First, single colonies were inoculated in TPGY and incubated for 14 h. Then, this pre-culture was diluted hundredfold in fresh TPGY and plated, and this point was defined as time 0. Already at time 0, 2.3 +/- 0.4 log spores/ml could be detected for the ClosTron mutant while spore counts of the wild type strain were under the detection limit at this time point (< 1 log spores/ml). Despite this difference in spore counts, the total cell counts did not show any significant difference, with 5.3 +/- 0.1 log cfu/ml and 5.5 +/- 0.2 log cfu/ml for the ClosTron mutant and parental strain respectively. Thus, a significant higher fraction of spores per total count was demonstrated for the ClosTron mutant, as shown in Table 4.3. Also after 10 h of growth, the spore fraction remains to be significantly higher for the *bontE*211a::CT mutant, while no significant difference is shown after 23 h and 40 h.

Table 4.3: The fraction of spores in cultures of the wild type strain and the *bontE*211a::CT mutant. Means +/- standard deviations are shown of experiments in triplicate. Significant differences in the spore fraction are indicated with * ($P < 0.05$).

Time (h)	NCTC 11219			*bontE*211a::CT	
0	$<3.79 \times 10^{-5}$	$+- 2.13 \times 10^{-5}$	*	1.33×10^{-3}	$+- 1.12 \times 10^{-3}$
10	1.20×10^{-5}	$+- 6.16 \times 10^{-6}$	*	1.78×10^{-3}	$+- 2.96 \times 10^{-4}$
23	5.26×10^{-5}	$+- 8.44 \times 10^{-5}$		1.00×10^{-3}	$+- 8.66 \times 10^{-4}$
40	4.22×10^{-3}	$+- 5.01 \times 10^{-3}$		6.07×10^{-3}	$+- 1.29 \times 10^{-3}$

In a variant of this experiment, colonies were picked up from a plate and directly inoculated in TPGY broth in triplicate, without making a preculture in TPGY. After 10 h of growth, the

spore counts were determined. At the start of inoculation, t = 0, no spores were detected for both strains (< 1 log spores/ml). 10 h after inoculation, the ClosTron mutant already showed 3.0 +/- 0.2 log spores/ml, while spore counts of the wild type strain were still under the detection limit at this time point. 14 h after inoculation, 4.0 +/- 0.3 log spores/ml could be detected for the mutant whereas 1.6 +/- 0.5 log spores/ml were present in the wild type culture, thus again a significant higher spore number was shown for the mutant. Although no total cell counts were recorded in this second experiment, the results are in line with the first experiments, and indicate that the ClosTron mutant sporulates earlier during growth than the wild type strain.

Spore heat resistance. In the analysis of spore heat resistance, we incorporated lysozyme in the recovery medium because this is known to increase the number of survivors of gIICb spores (see 1.7) [186,195]. This is because the cortex hydrolases which are required for spore germination are very heat sensitive, but can be substituted by exogenous lysozyme. The inactivation curves at 90 °C and 93 °C showed a biphasic trend, with an initial rapid decrease of about 2 – 2.5 log, followed by a slower log-linear decrease (Fig. 4.5). This is in line with previous reports, and has been attributed to the existence of a lysozyme-impermeable and -permeable fraction in the spore population [196]. The D-values were calculated only from the log-linear part, corresponding to the presumed lysozyme-permeable fraction. No differences were observed in the $D_{90°C}$ and $D_{93°C}$ between the three strains, suggesting that spore heat resistance is not affected by inactivation of *bont/E*. In addition, we chose to report heat resistance without lysozyme in the recovery medium. This reduces the apparent heat resistance of the spores, and treatments were therefore performed at 70 °C and 73 °C. Inactivation was log-linear in this case (Fig. 4.6), and presumably correlates with the inactivation of the spore cortex hydrolases. The $D_{70°C}$ of the two mutants was slightly but significantly lower than that of wild type, but no difference was found between the $D_{70°C}$ of both mutants. In contrast, the $D_{73°C}$ was not different between the three strains. Together, these results indicate that spore heat resistance is unaffected in the mutants, except for a small difference at 70 °C. Since this difference was observed both in the insertion and deletion mutant, it is unlikely to result from an accidental mutation unrelated to the knockout of the *bont/E* gene. One possible explanation is that the effect is due to a polar effect on one or more genes upstream or downstream of *bont/E*. However, given the gene context as shown in Fig. 4.2, it is difficult to imagine how the same polar effect could exist in both mutants. Finally, it cannot be excluded that the difference is a direct consequence of the abolition of toxin production. For example, it has been recently demonstrated that there is a regulatory link

between sporulation and toxin production in gIICb strain Beluga via the Spo0A gene product [23].

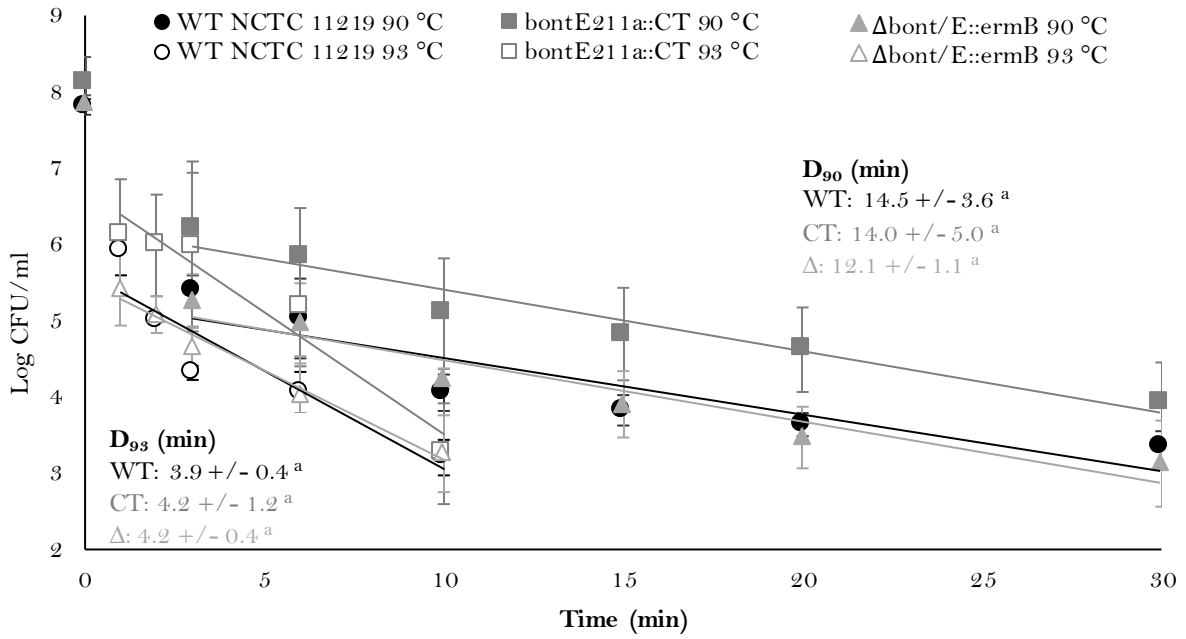

Fig. 4.5: Heat inactivation of spores of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* at 90 °C and 93 °C. Means +/- standard deviation of three independent experiments are shown, as well as average D-values +/- standard deviation at 90 °C and 93 °C derived from the linear part of the curve. A common letter superscript at the same temperature indicates that there is no significant difference.

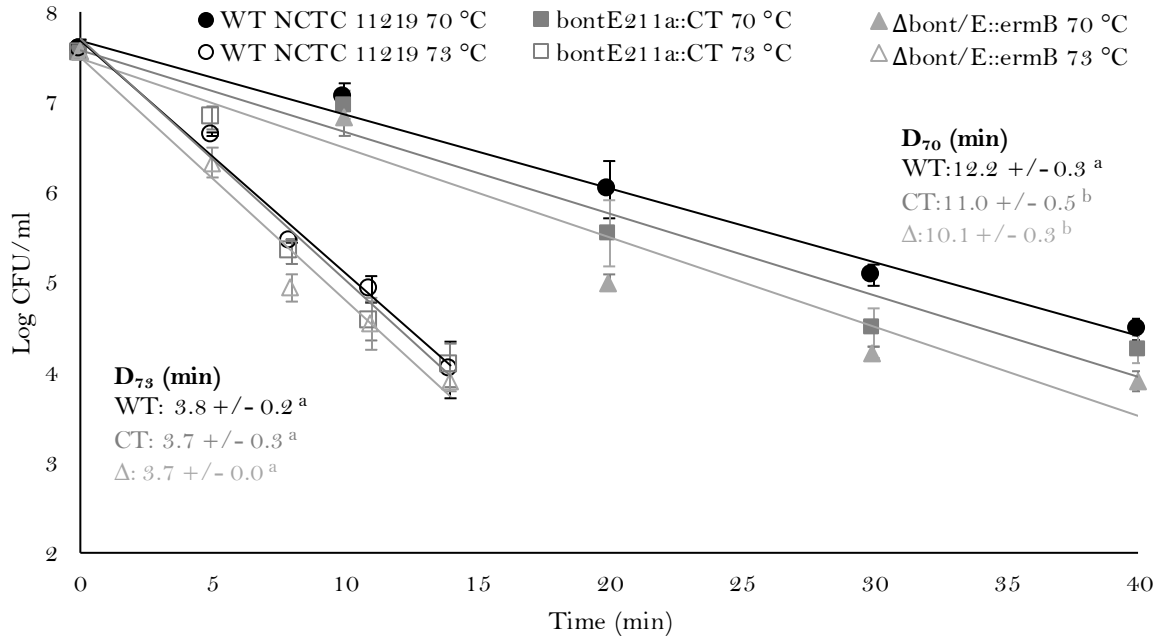

Fig. 4.6: Heat inactivation of spores of wild type NCTC 11219, NCTC 11219 *bontE*211a::CT and NCTC 11219 Δ*bontE*::*ermB* at 70 °C and 73 °C. Means +/- standard deviation of three independent experiments are shown, as well as average D-values +/- standard deviation at 70 °C and 73 °C. A common letter superscript at the same temperature indicates that there is no significant difference. Since tailing occurred after 40 min at 70 °C and after 15 min at 73 °C, the corresponding data is not shown or used.

4.4 Conclusion

In this chapter, two strategies were employed for obtaining a gIICb surrogate in which the *bont/E* gene is inactivated. Besides the well-known ClosTron system which generates insertional knockouts, we additionally developed a new approach based on double homologous recombination for the replacement of *bont/E* with an *ermB* cassette. Growth under unstressed and stressed conditions, spore yield and spore heat resistance of both mutants, compared with the wild type NCTC 11219, were unaffected, except for two properties where small differences were noted. Heat resistance of the spores at 70 °C was slightly lower for both mutants, and growth of the deletion mutant in 2.3 % NaCl was faster than for the other strains. In addition, an early onset of sporulation was demonstrated for the ClosTron mutant. In this mutant, the fraction of spores was much higher ($\sim 10^{-3}$) than the fraction in the wild type strain ($\sim 4.10^{-5}$) at time points where the cultures are reaching stationary phase.

The reason for these changes is currently unclear, and it could be of interest to investigate whether they are related to the loss of toxin production or to random mutations that have occurred during strain construction. However, since the deviations in the mutants are small (except for the early sporulation onset in the ClosTron mutant) compared to the natural strain-to-strain variation within gIICb, the nontoxic strains will be useful to investigate the safety of novel food processing and preservation techniques and for food challenge studies. Furthermore, the method for gene deletion developed in this work is a novel tool to construct nontoxic derivatives of type B and F gIICb strains as well as to inactivate other genes in gIICb and other clostridia.

Chapter 5

Validation of nontoxigenic mutants of group II *C. botulinum* type E strain NCTC 8266, and a deeper look into the nontoxigenic mutants of NCTC 11219 and NCTC 8266 by whole genome sequencing

5.1 Introduction

The previous chapter described the construction of two nontoxigenic mutants of gIICb strain NCTC 11219. The ClosTron system [187] was used for creation of a *bont/E* insertion mutant, whereas a new knockout strategy was developed which enabled the construction –for the first time- of a *bont/E* deletion mutant. While most phenotypical characteristics of the mutants were identical to the parental strain, also some unexpected differences were noted. Heat resistance of the spores at 70 °C was slightly lower for both mutants, and growth of the deletion mutant in 2.3 % NaCl was faster than for the other strains. In addition, an early onset of sporulation was demonstrated for the ClosTron mutant.

In this chapter we aimed to investigate whether these changes are related to the ClosTron insertion in, or the gene replacement of, the *bont/E* gene, or are due to random mutations that may have occurred at other genomic locations during mutant construction. To investigate the first option, both knockout strategies were repeated on a second strain, *C. botulinum* NCTC 8266. The phenotypical characteristics of the constructed NCTC 8266 mutants are presented in comparison with the wild type strain NCTC 8266. If phenotypical alterations could be observed that were also seen for the NCTC 11219 mutants, this could suggest that the inactivation of BoNT plays a role in these changes. In Chapter 3 the genome sequences of NCTC 11219, NCTC 8266 and NCTC 8550 were determined and this showed that NCTC 8266 and NCTC 8550 are highly similar (99 % identity) whereas the genome of NCTC 11219 is more different (93 % identity), e.g. it is larger in size and contains more prophages. Therefore, strain NCTC 8266 was chosen arbitrarily. The four sequence contigs of NCTC 11219 show respectively 93 %, 82 %, 52 % and 89 % query coverage with the WGS of NCTC 8266, all with 99 % identity (according to BLAST).

The construction of nontoxigenic derivatives of a second gIICb strain is also relevant for food challenge studies since these studies optimally require inclusion of a strain cocktail of the pathogen or pathogen surrogates to account for strain-to-strain variability.

In a second approach, WGS analysis was performed on the NCTC 11219 mutants and on the newly constructed NCTC 8266 Δ*bont::ermB* mutant to detect mutational events that could possibly explain the observed phenotypical changes.

5.2 Materials and Methods

5.2.1 Bacterial strains, construction of plasmids and mutants

C. botulinum type E NCTC 8266 was obtained from Wetenschappelijk Instituut Volkgezondheid (WIV, Brussels, Belgium), that obtained the strain from the National Collection of Type Cultures (Public Health England). Clostridial and *E. coli* cultures were grown as described in Chapter 4, see 4.2.1. All insertion and deletion knockout protocols that were used for the creation of the NCTC 8266 nontoxigenic mutants, were performed exactly as described in 4.2.3 - 4.2.6 for the NCTC 11219 strains. Because the upstream locus (1080 bp) of *bont/E* differs in only one base pair between NCTC 11219 and NCTC 8266 while the downstream locus is identical (1261 bp), the same plasmid (pMTL8415_Δ*bont*, containing the up- and downstream loci) was used to delete *bont/E* in NCTC 8266. Also the same ClosTron plasmid that was used for *bont/E* insertion in NCTC 11219, was employed to create the insertion mutant of NCTC 8266 since an identical targeting sequence is present. On the contrary, to delete *pyrE* and to finally restore *pyrE*, new plasmids were constructed, because the 2955 bp *pyrE* locus in NCTC 8266 (containing up- and downstream loci of *pyrE* as well as *pyrE* itself) contains 16 base pairs that are different from the *pyrE* locus in NCTC 11219. Therefore, the pMTL84151_*pyr5'pyr3'* and pMTL84151_WT*pyr* plasmids were constructed as described in 4.2.3 (with the same primers), by using the gDNA of NCTC 8266 for amplification of the homologous loci.

5.2.2 Phenotypical analysis of wild type NCTC 8266 and *bont/E* mutants

All experiments assessing stressed growth (NaCl, pH, low temperature), spore yield and heat resistance were performed in an identical manner as for NCTC 11219, described in 4.2.8, and microscopic examination was performed as explained in 4.2.2. In contrast, unstressed growth was determined differently. While a plate counting assay was used for growth of the NCTC 11219 strains, growth of the NCTC 8266 strains was assessed by following OD_{630} in time. More specifically, single colonies were inoculated in triplicate in TPGY broth, after which the overnight grown cultures were diluted hundredfold in the same medium in a 96-well microtiter plate. The plate was sealed with an oxygen impermeable cover foil (EASYseal^{Tm}, Greiner, Germany) and subsequently the OD_{630} was measured outside the anaerobic cabinet at 30 °C with regular shaking every 15 min.

5.2.3 Whole genome sequencing of the mutants

To identify all mutational changes that eventually occurred during mutant construction, whole genome sequencing was performed on an Illumina MiSeq sequencer.

The gDNA from mutants from two *C. botulinum* backgrounds (NCTC 11219 and NCTC 8266) was isolated from overnight cultures using the GeneJET Genomic DNA purification kit (Thermo Scientific). To obtain high-quality gDNA, formaldehyde was used for fixation of cells prior to isolation [163]. This step is essential to avoid DNA degradation by abundant extracellular DNase activity in gIICb cultures. DNA purity and concentration was assessed by Nanodrop analysis, gel electrophoresis and Qubit (Thermo Scientific) analysis. Paired-end libraries were constructed using the NEBNext Ultra gDNA library prep protocol with an average insert size of 240 bp, and analysed on the Agilent BioAnalyzer (VIB Nucleomics Core) resulting in on average 1.2 million reads per sample.

In collaboration with Bram Van den Bergh (Centre of Microbial and Plant Genetics, KU Leuven), reads were analysed with Qiagen's CLC Genomics Workbench (v.7) (CLC Bio), and included standard quality control, read trimming and filtering (reads < 15 nucleotides were discarded, quality score limit = 0.01, ambiguous nucleotides trim limit=2), read mapping to *C. botulinum* references (NCBI accession numbers CP010520 (NCTC 8266) and JXMR00000000 (NCTC 11219), using parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8). Lists of mutations were obtained combining CLC tools: 'Fixed Ploidy Variant Detection' for point mutations and small INDELs and 'Coverage Analysis' and 'InDels and structural variants' for larger structural rearrangements. All mutations were manually inspected in the mapping.

5.3 Results and Discussion

We have constructed nontoxigenic mutants of strain NCTC 8266 in an identical manner as described for NCTC 11219 (see 4.2.3 - 4.2.6). The same ClosTron plasmid was employed for insertion mutagenesis as the one used in Chapter 4, because of an identical targeting sequence. In addition, the same *bont/E* deletion plasmid was used in NCTC 8266 as the one that was used in NCTC 11219, because of the highly similar up- and downstream loci (only 1 base pair difference). PCR and sequence analysis confirmed *bont/E* insertion and deletion in the *bontE*211a::CT and Δ*bontE::ermB* mutant respectively.

Here, the phenotypic characteristics of the insertion and deletion mutant are documented in comparison with the wild type strain NCTC 8266. In Chapter 4 it was demonstrated that (i) the NCTC 11219 deletion mutant shows a faster growth at 2.3 % NaCl than the other NCTC 11219 strains, (ii) the ClosTron mutant starts sporulation more early than the wild type strain and (iii) heat resistance of the spores at 70 °C was slightly lower for both mutants. If the same alterations could be observed in the NCTC 8266 nontoxigenic mutants, this would suggest that the inactivation of BoNT plays a role in these changes. Furthermore, whole genome analysis was performed on three mutants to identify all mutational changes that have been introduced during mutant construction.

5.3.1 Phenotypical analysis of wild type NCTC 8266 and *bont/E* mutants

Unstressed growth. The growth curves of the mutants and the wild type at 30 °C showed very similar patterns (Fig. 5.1). The DMFit software was used to determine the maximum growth rate μ_{max}, the lag phase and the upper asymptote y_{end}. No significant differences existed between the wild type, the ClosTron and the Δ*bontE* mutant ($p > 0.05$).

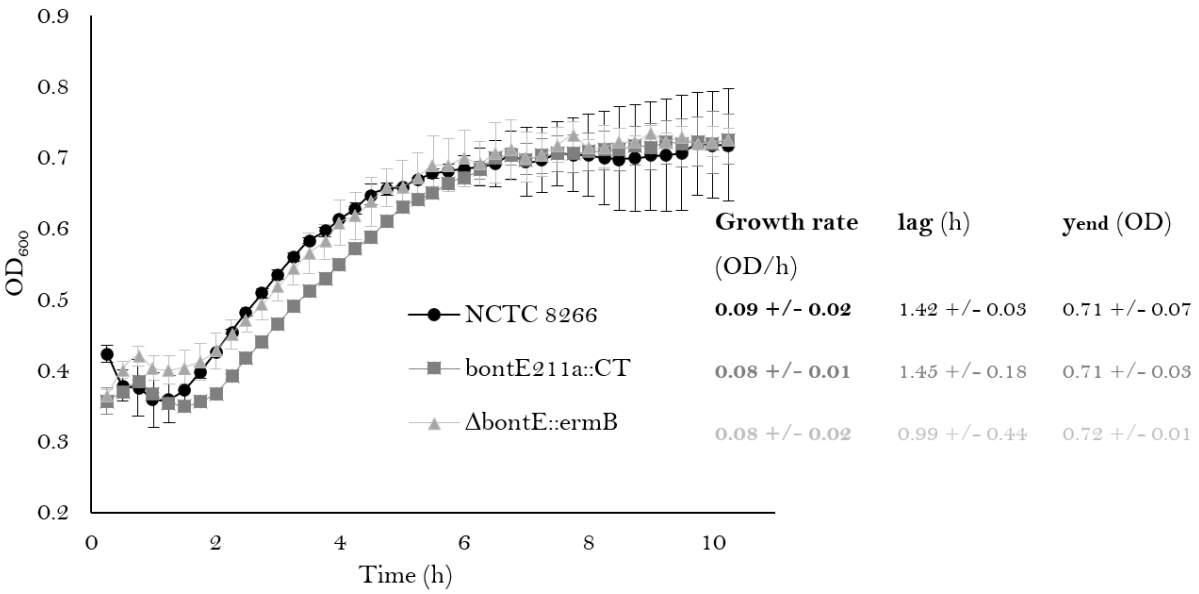

Fig. 5.1: Growth in TPGY broth at 30 °C of wild type NCTC 8266, NCTC 8266 *bontE*211a::CT and NCTC 8266 Δ*bontE*::*ermB* determined by OD. Means +/- standard deviations are shown from three independent experiments. The growth curves were modeled with DMFit to determine the growth rate, lag and upper asymptote y_{end}. No significant differences were found between the three strains ($p > 0.05$).

Stressed growth. In analogy with Chapter 4, a quantitative comparison of growth under acidic, NaCl and low temperature stress was performed by assessing the time to colony formation on RCM plates. On acidified RCM, results of both mutants and wild type were identical in sixfold (as well as identical to the results of the NCTC 11219 strains). Single colonies of 1 mm were visible after 24 h at pH 5.7 and after 30 h at pH 5.5, while pinpoint

colonies appeared after 72 h at pH 5.2. No colonies were formed at pH 4.9 up to 5 days. The second stress condition tested, was growth under NaCl stress. In Chapter 4, unexpectedly a difference was observed at 2.3 % NaCl between the Δ*bontE*::*ermB* strain compared to the wild type NCTC 11219. Strain Δ*bontE*::*ermB* formed clear 1 mm colonies after 24 h (in sixfold) while those of the wild type were pinpoint sized, and reached 1 mm only after 48 h (in sixfold). In contrast, no differences were seen between the NCTC 8266 and the mutants. All strains showed clear colonies on RCM containing up to 2.1 % NaCl after 24 h (in sixfold), while 48 h was needed to form clear colonies on 2.3 %. No strains formed colonies on 2.5 % NaCl.

The third condition tested, was growth at low temperature. Single colonies grown at 12 °C on RCM were restreaked in sixfold on RCM, then incubated at 12 °C and 8 °C, and the time to formation of colonies (1 mm) was registered. As opposed to the data for acid and NaCl stress, the time to colony formation at low temperature showed variability among the six replicates as well as between the strains, as indicated in Table 5.2. A logistic regression model was fit on this data. This model showed that the ClosTron strain was significantly different from the wild type at both temperatures ($P < 0.05$), whereas no difference was shown for the deletion strain ($P = 0.35$ (8°C); $P = 1$ (12 °C). The ClosTron mutant demonstrates a clear deficiency to grow at 8 °C, with only one of six replicates that showed colony formation (even after 17 days).

Table 5.2: Growth at 12 °C and 8 °C of wild type NCTC 8266, NCTC 8266 *bontE*211a::CT and NCTC 8266 Δ*bontE*::*ermB* on RCM plates. Single colonies were restreaked in sixfold and the time to formation of 1 mm colonies was noted.

	Number of plates showing single colonies						
	at 12 °C			**at 8 °C**			
Days (d)	**3 d**	**4 d**	**5 d**	**6 d**	**7 d**	**8 d**	**9 d**
NCTC 8266	5/6	6/6	6/6	5/6	6/6	6/6	6/6
*bontE*211a::CT	0/6	3/6	6/6	0/6	1/6	1/6	1/6
Δ*bontE*::*ermB*	5/6	6/6	6/6	3/6	4/6	6/6	6/6

Spore production. Sporulation was performed in a two-phase sporulation medium and observed regularly with phase-contrast microscopy. Spore formation started within 72 h for all strains. Spore counts were determined after six days by plate counting after inactivation of the vegetative cells at 65 °C for 10 min. No differences were observed in spore yield between wild type NCTC 8266, *bontE*211a::CT and Δ*bontE*::*ermB* ($p > 0.05$). Spore crops used for the heat treatments at 90 °C/93 °C had a yield of 8.7 +/- 0.4 log CFU/ml, 8.5 +/- 0.3 log CFU/ml and 8.7 +/- 0.3 log CFU/ml, respectively. The same crops were used for the heat treatments

at 70 °C/73 °C, but because this experiment was performed a few months later, the counts at t = 0 are slightly lower due to spontaneous germination during storage: 8.3 +/- 0.1 log CFU/ml, 8.3 +/- 0.1 log CFU/ml, 8.5 +/- 0.1 log CFU/ml respectively.

In Chapter 4, the onset of sporulation was determined in more detail for the NCTC 11219 *bontE*211a::CT mutant because we observed with phase-contrast microscopy that unusual high numbers of spores were already present in overnight grown cultures. However, this was not observed for the NCTC 8266 mutants and therefore, the onset of sporulation was not assessed in detail here.

Spore heat resistance. In the analysis of spore heat resistance at 90 °C and 93 °C, we incorporated lysozyme in the recovery medium because this is known to increase the number of survivors of gIICb spores. The inactivation curves at 90 °C and 93 °C showed a biphasic trend, with an initial rapid decrease of about 2.7 log, followed by a slower log-linear decrease (Fig. 5.3). This is in line with previous reports, and has been attributed to the existence of a lysozyme-impermeable and -permeable fraction in the spore population [88,196]. The D-values were calculated only from the log-linear part, corresponding to the presumed lysozyme-permeable fraction. No differences were observed in the $D_{90°C}$ and $D_{93°C}$ between the three strains. Furthermore, the $D_{70°C}$ and $D_{73°C}$ were determined without the addition of lysozyme. While in Chapter 4, a $D_{70°C}$ was demonstrated for both NCTC 11219 mutants that was slightly but significantly different from the wild type (Fig. 4.6), there was no difference shown here between the NCTC 8266 strains (Fig. 5.4).

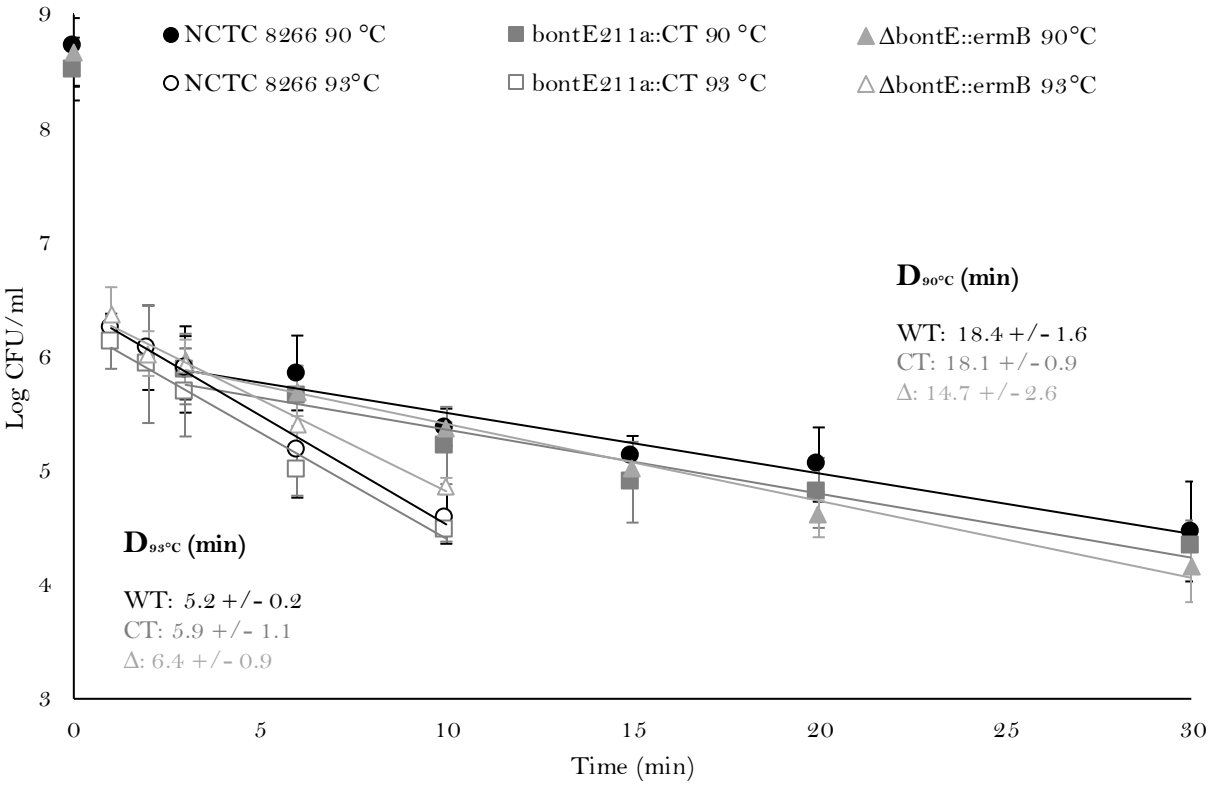

Fig. 5.3: Heat inactivation of spores of wild type NCTC 8266, NCTC 8266 *bontE*211a::CT and NCTC 8266 Δ*bontE*::*ermB* at 90 °C and 93 °C. Means +/- standard deviation of three independent experiments are shown, as well as average D-values +/- standard deviation at 90 °C and 93 °C derived from the linear part of the curve. No significant differences were shown between the strains (P value > 0.05).

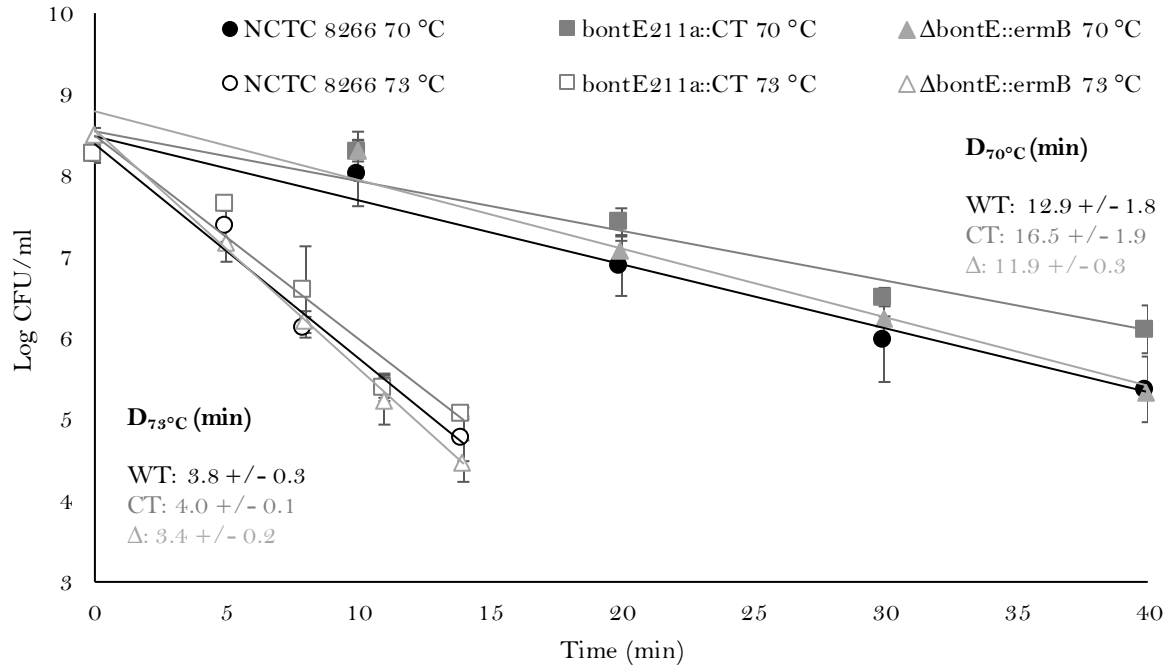

Fig. 5.4: Heat inactivation of spores of wild type NCTC 8266, NCTC 8266 *bontE*211a::CT and NCTC 8266 Δ*bontE*::*ermB* at 70 °C and 73 °C. Means +/- standard deviation of three independent experiments are shown, as well as average D-values +/- standard deviation at 70 °C and 73 °C. No significant differences were shown between the strains (P value > 0.05).

5.3.2 Whole genome sequencing of the mutants

To further investigate the altered phenotypes that have been described earlier for the nontoxigenic mutants of strain NCTC 11219 (Table 5.3), WGS analysis was performed to identify mutational changes that may have occurred during genome engineering. The NCTC 8266 deletion mutant did not show any altered phenotype, but it was included in the analysis as well. In contrast, the NCTC 8266 ClosTron mutant was not included, although it has a defect in growth at low temperature that would have been interesting to analyse. This is unfortunate in retrospect, and it will be interesting to sequence this strain in the future.

Table 5.3: Nontoxigenic mutants that were subjected to WGS analysis, and phenotypical differences with their corresponding wild type strain.

Mutant strain	Altered phenotype
NCTC 8266 Δ*bontE::ermB*	No changes detected
NCTC 11219 Δ*bontE::ermB*	Faster growth at 2.3 % salt; lower D-value at 70 °C
NCTC 11219 *bontE*a211::CT	Early sporulation onset; lower D-value at 70 °C

Sequencing was performed on an Illumina Miseq sequencer, yielding around 1.2 million reads of 150 bp for all mutants with 47-, 49- and 52-fold total coverage of respectively NCTC 8266 Δ*bontE::ermB*, NCTC 11219 Δ*bontE::ermB* and NCTC 11219 *bontE*211a::CT. Table 5.4 demonstrates all mutational changes (variant frequency > 60 %) introduced in the nontoxigenic mutants, in comparison with the parental strains NCTC 8266 and NCTC 11219.

WGS NCTC 8266 *ΔbontE::ermB*

Although no phenotypical alterations of the NCTC 8266 *ΔbontE::ermB* mutant were found, the genome analysis showed four variations in comparison with the parental strain NCTC 8266. It should be noted that the SNP that was found in ST13_05335, encoding a protein annotated as peptidase M27, was an expected mutation. This locus tag ST13_05335 is the *ntnh* gene which is located directly upstream the deleted *bont/E* (Fig. 4.2). Because the *bont/E* flanking loci of the NCTC 8266 and NCTC 11219 parental strains differed in one nucleotide, and since the same deletion plasmid (on which these homologous regions were located) was used for deletion of *bont/E*, this SNP was introduced on purpose. Further, three other variations were found which were unexpected. Frameshift mutations were identified in genes encoding a predicted thymidine kinase and a GGDEF-domain containing protein, and an amino acid change has occurred in a predicted membrane protein. However, the possible impact of these mutations on physiology was not studied further.

WGS NCTC 11219 *ΔbontE::ermB*

Surprisingly, the genome analysis of NCTC 11219 *ΔbontE::ermB* showed a high number of mutations, with multiple large deletions (~ 18 kb, 20 kb and 51 kb). This was quite unexpected, since it was concluded from the phenotypical analysis that the mutant did not reveal major differences compared to the wild type strain, except for a small difference in spore heat resistance at 70 °C and in growth at 2.3 % NaCl. The 18 kb deletion encompasses predicted restriction enzymes, which could possibly explain another observation that was made during several experiments, but that was not documented quantitatively: the NCTC 11219 Δ*bontE::ermB* mutant is a much better acceptor in conjugation experiments than the wild type strain. In general, conjugation of plasmids to the NCTC 11219 wild type strain occurred with extremely low frequency. It is therefore possible that a colony was picked up in conjugation experiments in which the 18 kb region was deleted, resulting in a better mating acceptor. However, this is only speculation and has not been studied further. The other large deletions are phage-like regions, encompassing one intact and two incomplete prophage regions according to PHAST.

Table 5.4: Mutational changes introduced during mutant construction. (fs: frameshift; *: stop codon introduced; Met1?: mutated start codon)

	Mutation	Predicted protein	Amino acid change	Location [1]	Locus tag [1]	Accession nr. [1] (NCBI)
NCTC 8266	AAATC deletion	Thymidine kinase	Lys94fs	468617	ST13_02225	CP010520
Δ*bontE::ermB*	A insertion	GGDEF containing protein	Gln55fs	572028	ST13_02755	CP010520
	T→C	Peptidase M27	Ile1131Val	1165722	ST13_05335	CP010520
	C→T	Membrane protein	Asp127Asn	2827151	ST13_12815	CP010520
NCTC 11219	18 kb deletion	Multiple proteins [2]		442922-461386	SR42_14245 -SR42_14305	JXMR01000002
Δ*bontE::ermB*	51 kb deletion	Prophage like region [3]		15573 - 66817	SR42_15045-SR42_15415	JXMR01000003
	20 kb deletion	Prohage like region [4]		10 000 - 30 000	SR42_15790-SR42_15900	JXMR01000004
	67 bp deletion	DNA-binding transc. regulator		2310781-2310848	SR42_10440	JXMR01000001
	C→A	Chemotaxis protein CheD	Ser29*	1892398	SR42_08525	JXMR01000001
	T→A	Chemotaxis protein CheA	Val639Glu	1896625	SR42_08540	JXMR01000001
	G→A	ATPase	Gly427Glu	1474459	SR42_06595	JXMR01000001
	A→C	Thymidine kinase	Lys56Asn	1577590	SR42_07135	JXMR01000001
	G→T	Acetyl-CoA carboxylase biotin carboxyl carrier	Lys52Asn	2268595	SR42_10275	JXMR01000001
	G→A	Hypothetical protein	Pro67Ser	709267	SR42_03175	JXMR01000001
	T deletion	Cell wall binding protein	Ile133fs	688756	SR42_03090	JXMR01000001
	G→T	LacI transcr. regulator	Met1?	337323	SR42_01725	JXMR01000001
	CTT deletion	GTP binding protein YchF	Glu245Val (- Glu)	93940	SR42_16260	JXMR01000004
NCTC 11219	T→A	GGDEF containing protein	Leu210Phe	116622	SR42_16335	JXMR01000004
***bontE*a211::CT**						

[1] in parental strain ; [2] type I restriction enzyme EcoKI subunit R, repressor LexA, restriction endonuclease, HNH endonuclease, DNA helicase, hypothetical proteins ; [3] According to Phast, encompassing one intact and one incomplete prophage. XRE family transcriptional regulator, Cro/Cl family transcriptional regulator, transcriptional regulator, cell wall binding protein, membrane protein, UviB-like protein, tail fiber protein, Xkdt protein, phage like element, N-acetylmuramoyl-L-alanine amidase, prophage protein, ABC transporter ATPase, guanosine polyphosphate pyrophosphohydrolase, terminase, DNA primase, phage protein, ERCC4-type nuclease, AbrB family transcriptional regulator, hypothetical proteins ; [4] According to Phast, encompassing an 11 kb questionable prophage region. Phage portal protein, terminase, DNA-directed RNA polymerase sigma-70 factor, site-specific integrase, peptidase M20, nitroreductase, DNA helicase, HNH endonuclease, ParB protein, cellulose biosynthesis protein BcsQ, transcriptional regulator, endonuclease, ImmA/IrrE family metallo-endopeptidase, integrase, hypothetical proteins.

Deletions of such large loci will presumably have an impact on the physiology of NCTC 11219 *ΔbontE::ermB*. However, according to BLAST, it seems like these large loci are not well conserved among gIICb strains. We are currently investigating this in more detail by evaluating all available gIICb sequences.

Besides these large deletions, a number of other mutations were found in the genome of NCTC 11219 Δ*bontE::ermB*. For example, two mutations were found in the genes encoding the chemotaxis proteins CheA and CheD, and we therefore attempted to detect a modified chemotaxis behaviour. However, the standard test to assess swimming motility in 0.3 % agar could not be used because the cells produced too much gas and thereby disrupted the agar medium. In addition, swarming motility on the surface of 0.4 % and 0.6 % agar could not be observed, not even for the wild type strain.

WGS NCTC 11219 *bontE*a211::CT

The NCTC 11219 ClosTron mutant differs from the wild type strain in that it forms spores in rich growth medium more rapidly. The genome analysis of NCTC 11219 *bontE*a211::CT identified only one SNP, located in a GGDEF-domain containing protein. These proteins act as diguanylate cyclases which synthesize cyclic dimeric guanosine monophosphate (c-di-GMP), a bacterial intracellular signaling molecule. Interestingly, there was also a mutation in a gene encoding a different GGDEF-domain containing protein in the NCTC 8266 Δ*bontE::ermB* strain (Table 5.4). The NCTC 11219 and NCTC 8266 genomes encode six GGDEF-domain containing proteins, and the mutated genes (SR42_16335 and ST13_02755) encode proteins that share only 32 % sequence identity over a region encompassing 46 % of the entire sequence. The mutations are not located in the predicted GGDEF-domain of the proteins. To further investigate the effect of this mutation in the NCTC 8266 Δ*bontE::ermB* mutant, the onset of sporulation was studied in comparison with the wild type strain. Remarkably, instead of an early sporulation onset, the NCTC 8266 *ΔbontE::ermB* showed a delayed onset. While the NCTC 8266 wild type strain produced 2.5 +/- 0.3 log spores/ml 14 h after colony inoculation in TPGY broth, the NCTC 8266 Δ*bontE::ermB* showed no detectable spore count (< 1 log spores/ml) after 14 h and after 18 h, but only after 21 h (1.7 +/- 0.3 log spores/ml). However, because NCTC 8266 *ΔbontE::ermB* has three other mutations, this delay in sporulation cannot be directly linked to the mutated diguanylate cyclase without further experiments.

The potential role of diguanylate cyclases in the onset of sporulation would be very interesting to investigate further. The messenger c-di-GMP has been mostly studied in Gram-negative

bacteria and is known to be a key player in regulation of all kinds of lifestyle changing events, such as the transition between motility and sessility, persistence, biofilm formation, cell differentiation, changes in adhesiveness and virulence gene expression [197–199]. In comparison, very little is known about c-di-GMP signaling in Gram-positive bacteria. It has been shown that elevated levels of the secondary messenger inhibit motility in *C. difficile* and *B. subtilis* [200,201]. Moreover, very recently a regulatory link between c-di-GMP and sporulation was for the first time addressed in *B. thuringiensis* [202]. Mutants were constructed in *cdg* genes, which encode enzymes putatively involved in cyclic diguanylate synthesis and/or break-down. Although *cdgC* and *cdgJ* were predicted to encode inactive proteins because they carry a degenerate GGDEF motif, their inactivation showed a small but statistically significant change in sporulation. Inactivation of *cdgC* resulted in 2.5-fold higher sporulation efficiency, which was assessed after 20 h of growth. Deletion of *cdgJ*, in contrast, delayed sporulation with no spores detected after 20 h. In another recent study , it was shown that a decreased c-di-GMP level delays sporulation of *Myxococcus xanthus*, and it was suggested that a certain threshold level of c-di-GMP is essential to start sporulation [203]. In contrast, studies with *Streptomyces* indicated that excessive levels of c-di-GMP inhibit certain sporulation genes [204].

Although fragmentary and not conclusive, these data in different sporulating bacteria suggest a role for c-di-GMP signaling in sporulation that would be an interesting topic for future research.

5.4 Conclusion

As was done for *C. botulinum* NCTC 11219 (Chapter 4), a *bont/E* insertion and deletion mutant were constructed in strain NCTC 8266. In this chapter, the characterization of these two nontoxigenic mutants has been presented. Because the NCTC 11219 mutants showed some changes in phenotypical properties in comparison with the wild type strain (Table 5.3), we investigated whether these changes could be related with the inactivation of BoNT. This did not seem to be the case, since similar changes could not be seen in the NCTC 8266 mutants. The NCTC 8266 Δ*bontE*::*ermB* mutant was shown to have identical characteristics as the wild type strain for stressed and unstressed growth, spore yield and spore heat resistance. Therefore this strain is a very good candidate to be used as a safe surrogate organism. In contrast, the insertion mutant demonstrated an altered growth at lower temperature (8 °C and 12 °C).

In a second approach, we investigated whether random mutations were introduced during strain construction of the NCTC 11219 mutants, and whether these could explain the observed phenotypical changes. Therefore, WGS analysis was performed on both NCTC 11219 mutants, as well as on NCTC 8266 Δ*bontE*::*ermB*. The analysis showed a high number of mutations in NCTC 11219 Δ*bontE*::*ermB*, whereas the NCTC 8266 Δ*bontE*::*ermB* only showed four variations (Table 5.4). The gene replacement strategy comprises multiple steps (deletion of *pyrE*, double recombination with flanking loci of *bont/E*, restoration of *pyrE*), which increases the chance for mutations to occur. However, since both mutants were constructed in exactly the same way, the knockout strategy that was employed is not necessarily accountable for the high mutational rate in NCTC 11219 compared to NCTC 8266. However, NCTC 11219 contains more prophage regions than NCTC 8266. Such regions are known to be less stable and it is exactly in these regions that major deletions occurred. Although we concluded in Chapter 4 that NCTC 11219 Δ*bontE*::*ermB* is a good candidate for challenge testing, the WGS analysis showed that it should be kept in mind that this strain might react differently from the wild type in not yet tested conditions, due to the presence of multiple mutations. Next to phenotypical characterization, WGS analysis is thus an important tool in the validation of nontoxigenic mutants to be used as surrogates in food challenge studies.

Further, the early onset of sporulation that was observed in the NCTC 11219 ClosTron mutant, could tentatively be linked to a mutated diguanylate cyclase because this was the only mutation found in this strain. Diguanylate cyclases are involved in the synthesis of the

bacterial intracellular signaling molecule c-di-GMP. Although c-di-GMP is known to be a key player in regulation of all kinds of lifestyle changing events, a regulatory link with sporulation has only been demonstrated very recently by Fagerlund et al. (2016) [202]. In this study, a knockout of a diguanylate cyclase resulted in a 2.5-fold higher sporulation efficiency after 20 h of growth. In comparison, the sporulation efficiency reported in Chapter 4 for the NCTC 11219 ClosTron mutant was increased about hundredfold compared to the wild type strain. Further study of this mutant should clarify the role of c-di-GMP in sporulation onset in gIICb, thus providing an interesting novel insight in the complex developmental program of bacterial sporulation. Moreover, since there is evidence that BoNT toxin production is linked to sporulation in *C. botulinum*, c-di-GMP and the early sporulation phenotype may also have important implications for food safety.

Chapter 6*

The inhibitory effect of carvacrol, *trans*-cinnamaldehyde, carrot seed essential oil and hop β-acids on germination of group II *C. botulinum* NCTC 11219 spores

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6.1 Introduction

gIICb strains are a major concern for the safety of mildly heat-processed refrigerated foods with long shelf life. Because gIICb strains are psychrotrophic, spores surviving mild heat treatment may subsequently germinate, grow out and produce toxin during refrigerated storage. Therefore, food producers have to ensure that the formulation of the food (pH, salt, preservatives,…) and the storage conditions (temperature, gas atmosphere,…) prevent multiplication of gIICb during the shelf life of these foods [67,69,75,76,205]. Increasing consumer demands for high-quality, natural foods with minimal preservatives challenges the food industry regarding botulinum safety, because the effectiveness of emerging nonthermal processing techniques and biopreservatives against this pathogen are poorly documented [69,75].

Natural additives have become very popular to preserve foods. Essential oils (EOs) derived from spices and other plants are gaining a wide interest as 'natural' antimicrobial preservatives in the food industry that can replace traditional chemicals like nitrite, benzoate and others, for which studies have increased the concern for adverse health effects [206]. It has been well established that the active components of these oils have a wide spectrum of antimicrobial activity against foodborne pathogens and spoilage bacteria [207]. Carvacrol and *trans*-cinnamaldehyde, the main active components in respectively oregano EO and cinnamon bark EO, are considered broad-spectrum antimicrobials that are effective against many Gram-positive and Gram-negative bacteria as well as fungi (*B. cereus*, *L. monocytogenes*, *E. coli*, *C. perfringens*, *S. aureus*, *Salmonella* strains, *Aspergillus* spp., *Candida albicans*, etc.) [208–212]. Due to their hydrophobic nature, it is suggested that these compounds interact with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular compounds [213–215]. A less studied oil is carrot seed EO, which is mainly composed of carotol and carotenol. The antibacterial activity of carrot seed EO against *S. aureus*, *B. cereus*, *E. coli* and *S. typhimurium* has been reported, and for carotol only an antifungal effect has been described so far [216–218]. Only few studies have documented the activity of natural compounds against *C. botulinum*. One notable exception is a patent application claiming the inhibitory effect of hop extracts on growth of *C. botulinum* (group I and II), *C. difficile* and *Helicobacter pylori*, even at concentrations as low as 1 ppm [219]. The antimicrobial properties of hop extracts have also been documented against many other Gram-positives (i.e. *Listeria*, *Bacillus*, *Staphylococcus*), while only negligible to moderate activity has been demonstrated for Gram-negative bacteria (*E. coli*, *S. enterica*) [220–223]. Hop extracts contain high amounts of α- and β-acids (humulones and lupulones), with β-acids having the strongest antibacterial activity. It is postulated that undissociated hop acids incorporate into bacterial cell membranes and passively diffuse into

the cell. When the acids then dissociate because of a higher internal pH, the liberated protons acidify the cytoplasm, which leads to a dissipation of the transmembrane proton gradient, a decrease in proton motive force-driven uptake of nutrients, starvation and eventually to cell death [220,224]. However, the low reported minimal inhibitory concentration (MIC value) of hop acids for *C. botulinum* suggests that additional antimicrobial mechanisms may be involved in their action.

Unfortunately, limited data are available on the effect of these natural compounds on endospores. Our group recently reported on the antibacterial activity of eleven essential oils against both vegetative cells and spores of *B. cereus* [225]. All oils except one, i.e. carrot seed EO, had a inhibitory effect on *B. cereus* germination. The only data available on gIICb spores is published by Ismaiel & Pierson in 1990, where the effects of clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon oils on growth and germination of one gIICb type E strain and two gICb strains were studied [226]. In the type E strain, origanum oil was the most effective growth inhibitor, causing 98 % inhibition at ≥ 100 ppm. Pimento, clove, thyme and cinnamon followed in that order. At 50 ppm, depending on the oil between 20 % and 60 % growth inhibition was observed, while at 10 ppm no inhibition was observed anymore. The results were somewhat different for spore germination. At concentrations ≥ 100 ppm, all oils could effectively inhibit germination, with only 4 % germination relative to the control without oil. At 50 ppm, all oils still had a strong inhibitory effect (10 % of germination), except pimento and clove (40-60 % germination). At 10 ppm, only the oils of garlic and onion were still somewhat effective with less than 50 % germination.

In this chapter, we assessed the inhibitory effects of carvacrol, t-cinnamaldehyde, carrot seed EO and hop β-acids on nutrient and non-nutrient induced spore germination (see 2.2.1 and 2.2.2), using the nontoxigenic derivative from strain NCTC 11219 constructed in Chapter 4. Since spore germination necessarily preceeds vegetative cell growth and thus toxin production, the inhibition of germination by natural compounds could possibly be used to extend the shelf life of refrigerated foods by delaying gIICb multiplication and BoNT toxin formation.

6.2 Materials and Methods

6.2.1 Growth conditions, spore production and purification

For all experiments a nontoxigenic mutant of gIICb strain NCTC 11219 was used, designated as NCTC 11219 Δ*bont*::*ermB*, or briefly Δ*bont* (Chapter 4). The growth conditions and the media that were used for vegetative growth and sporulation are described in 4.2.1 and 4.2.2. The spores were harvested after six days of incubation at 30 °C, washed four times with 0.85 % sterile saline by centrifugation, concentrated fivefold and stored in saline at 4 °C. Spores were handled in ambient atmosphere, while incubation for outgrowth occurred in a Don Whitley DG250 anaerobic workstation (initial gas mixture 80 % N_2, 10 % CO_2, and 10 % H_2) using overnight deoxygenated media. Vegetative cells were always manipulated in the workstation.

6.2.2 Chemicals and stock solutions

The germinant mixture, further referred to as the gerMix, that was used to induce nutrient germination, was composed of L-alanine (Sigma), L-lactate sodium salt (Acros) and $NaHCO_3$ (Acros) at final concentrations of 100 mM, 50 mM and 50 mM respectively in 100 mM Tris-HCl buffer (pH 7.4). The gerMix solution was prepared freshly before use, as a twofold concentrated working solution. A 1 M stock solution of dodecylamine (Acros) was freshly prepared in ethanol, and further diluted in 100 mM Tris-HCl buffer to 6 mM (= twofold of working concentration). Stock solutions of 120 mM $CaCl_2$ (Chem Lab, Zedelgem, Belgium) and 120 mM DPA (Acros) were made in 100 mM Tris-HCl, and the pH of the DPA solution was readjusted to pH 7.5 with Trizma base (Sigma). The two solutions were then mixed in a 1:1 volume ratio and eventually further diluted in 100 mM Tris-HCl to achieve final Ca^{2+}-DPA concentrations of 60 mM, 50 mM, 30 mM and 20 mM.

Stock solutions of 8 mM carvacrol (Sigma) and 8 mM cinnamaldehyde (Acros) were prepared freshly in 2x gerMix or 2x dodecylamine, and further diluted in the same solution. A stabilized stock emulsion of 10 % (v/v) carrot seed essential oil (Anthémis Aromatherapie, Oosterstreek, The Netherlands) was made in 100 mM Tris-HCl buffer containing 0.2 % agarose and 2x gerMix or 2x dodecylamine. The agarose was used to accomplish a stabile emulsion of the oil, which was then further diluted in 2x gerMix or 2x dodecylamine to achieve the concentrations to be tested. The Beta Bio 45 % solution (Hopsteiner, Mainburg, Germany) contains 45 % (w/w) of the natural β-acids fraction of hops extract in propylene glycol. This solution was

further diluted in 2x gerMix or 2x dodecylamine to obtain the tested concentrations of the β-acids.

6.2.3 MIC on vegetative growth determined by the broth dilution method

The minimal inhibitory concentration (MIC) for vegetative cells was determined as the concentration that inhibits growth within 24 h at 30 °C by measuring optical density (OD). Different concentrations of carvacrol, cinnamaldehyde, carrot seed EO and hop β-acids in TPGY broth were added in 96-well microtiter plates. Then, stationary phase cultures were diluted and added to the compounds to achieve a final concentration of approximately 2.10^5 CFU/ml in the wells. Final concentrations of 4 mM, 2 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM of both carvacrol and cinnamaldehyde were tested (~ 615 to 19 ppm for carvacrol, and 503 to 16 ppm for cinnamaldehyde). Carrot seed EO was tested at 1 %, 0.5 %, 0.25 % and 0.125 % (v/v) (~ 10000 ppm to 1250 ppm) and hop β-acids at concentrations 0.001 %, 0.0005 %, 0.00025 %, 0.0001 %, 0.00005 %, 0.000025 % (~ 10 to 0.25 ppm). Positive (TPGY + cell suspension) and negative controls (TPGY) were included. Since a 10 % stock emulsion of carrot seed EO was used that contained 0.2 % agarose, the negative control here contained the same amount of agarose as 1 % carrot seed EO. The analysis was done with three independent cultures, and the MIC was defined as the concentration for which the OD_{600} value was not significantly different from the OD of the negative control, after 24 h incubation at 30 °C.

6.2.4 Nutrient and dodecylamine induced germination with/without inhibiting compounds

Spore crops were centrifuged (3400 x g, 10 min, 4 °C), resuspended in 100 mM Tris-HCl and heated for 10 min at 65 °C to inactivate any residual vegetative cells and spores that would have spontaneously germinated during storage, and also to activate dormant spores for germination. Hereafter a sample was taken from the suspension, diluted and plated on TPGY to determine the initial spore count N(t0). The remainder of the heat activated spore suspension was subdivided and added in a 1:1 volume ratio to 2x gerMix or 2x dodecylamine, with or without the tested inhibitory compounds at 2x concentration, and incubated for 1 h at 30 °C. Then, one part of the suspension was diluted and plated, designated N(t1-noHT), whereas the other part was again heat treated for 10 min at 65 °C before plating, N(t1-HT). Because the spores that have germinated within 1 h have lost their heat resistance, the difference in counts between N(t1-noHT) and N(t1-HT) corresponds to the amount of germinated spores. However, this can only be the case if counts of N(t0) are similar to N(t1-

noHT), demonstrating that no spore inactivation has taken place during the incubation, but only germination. There was never a significant reduction shown when comparing counts N(t0) with N(t1-noHT). Therefore, the following formula was used to determine the log germination.

$$\textbf{Log germination (log CFU/ml)} = \log_{10}(\textbf{N(t0) / N(t1-HT)})$$

Germination by plate counting was assessed after 1 h, because the germination experiment was conducted outside the anaerobic cabinet, and a longer incubation period could thus inactivate the germinated spores due to oxygen exposure. This would result in a lowered count of N(t1-noHT), not comparable anymore with counts of N(t0), making it impossible to distinguish inactivation by oxygen from inactivation by the compound.

All experiments were performed in triplicate with independent spore crops. The log germination in gerMix/dodecylamine with addition of a tested compound was determined relatively to the log germination in gerMix/dodecylamine alone for every spore crop. The germination assays with the hop β-acids were performed by Aurélie De Jong (Laboratorium of Food Microbiology, KU Leuven).

6.2.5 Ca^{2+}-DPA induced germination

The germination assay was performed as described for nutrient germination, with one small alteration. Ca^{2+}-DPA was used at 20 – 60 mM for germination experiments, but since a twofold concentrated solution of 120 mM cannot be made because of the limited solubility of Ca^{2+}-DPA, the spores were resuspended immediately in 60 mM, 50 mM, 30 mM and 20 mM Ca^{2+}-DPA, and the initial heat treatment to activate the spores was done in the presence of Ca^{2+}-DPA.

6.2.6 High pressure induced germination

Spore crops were centrifuged (3400 x g, 10 min, 4 °C), diluted tenfold and resuspended in 100 mM Tris-HCl and heated for 10 min at 65 °C. A part of the sample was plated as described before, to determine the initial spore count N(t0). Hereafter, the spore suspension was subdivided and transferred to 1 ml heat-sealed sterile polyethylene bags that were subjected to 200 MPa, 600 MPa, as well as to atmospheric pressure as the control. Pressure treatment was performed for 15 min at 30 °C in an eight-vessel HP device (HPIU-10 000, 95/, 1994, Resato, Roden, The Netherlands). The temperature of the vessels was controlled by a water circuit connected to a cryostat and a mixture of glycols (TR15, Weesp, The Netherlands) was used as pressure-transmitting liquid. After the pressure treatment, the spores were incubated

aerobically for 4 h at 30 °C at atmospheric pressure to allow a maximal germination response. Hereafter, the sample was heat treated (65 °C, 10 min) to determine the fraction of germinated spores by plating, with the same formula as described above.

6.2.7 DPA measurements

While germination assessment by plate counting was done after 1 h incubation to limit oxygen exposure, germination assessment by DPA measurements was done after longer germination times to maximize the germination degree. After 4 h of incubation, spores were centrifuged (12 000 x g for 5 min) to remove the spore pellet from the germinant solution, whereafter the supernatant was diluted tenfold in Tris-HCl buffer and stored at – 20 °C until DPA analysis. Because the spore crops are already diluted tenfold at the start of the high pressure experiments, the supernatant was not further diluted in this case. DPA measurement was based on formation of a fluorescent complex with Tb^{3+} as described before [227]. Hundred µl of the spore supernatant from – 20 °C was mixed with an equal amount of 20 mM $TbCl_3$ in a 96-well black microtiter plate (Greiner Bio-one, Vilvoorde, Belgium). Fluoresence measurements were done in a spectrofluorometer (Synergy Mx-biotek, VT, USA) with excitation and emission wavelengths of 270 and 545 nm, respectively. Background controls of the buffer, gerMix or dodecylamine with/without inhibiting compounds, were taken into account as well. The signal of these controls was substracted from the signal of the spore supernatant. Then, DPA release was expressed relative to the value obtained for the supernatant of spores that were subjected to 98 °C for 30 min, because these spores are assumed to have released their entire DPA depot [144]. The relative DPA release was determined as the proportion of DPA release in the presence of the tested inhibitory compound (together with gerMix or dodecylamine) to DPA release without the tested compound.

6.2.8 Microscopy

Spore suspensions were immobilized on thin pads of 1 % agarose in Tris-HCl buffer. Pictures were taken with a Ti-Eclipse inverted microscope (Nikon, Champigny-sur-Marne, France) equipped with a CoolLED pE-100 camera. Images were acquired using NIS-Elements (Nikon) and resulting pictures were further handled with open source software ImageJ.

6.2.9 Statistical analysis

All experiments were performed with three independent spore crops, and the two-tailed unpaired Student's t-test with a significance level of 0.05 was used to statistically assess the effect of inhibitors on germination and DPA release.

6.3 Results and Discussion

6.3.1 Germination induced by nutrient and non-nutrient germinants

It has been shown previously that gIICb spores germinate efficiently in response to L-alanine/L-lactate/$NaHCO_3$ (≥ 90 % or ≥1 log germination) [122]. Therefore, we used this combination, further in the text referred to as the gerMix, to test nutrient induced germination of NCTC 11219 Δ*bont* spores. This nontoxic mutant was chosen instead of NCTC 8266 Δ*bont* only because it was the first one constructed. Additionally, we analysed non-nutrient induced germination by exogenous Ca^{2+}-DPA, dodecylamine and high pressure (HP).

Fig. 6.1 shows germination of NCTC 11219 Δ*bont* spores by nutrient and non-nutrient inducers, based on loss of heat resistance (65 °C/10 min) and DPA release (not for Ca^{2+}-DPA induced germination). Exogenous Ca^{2+}-DPA (20, 30, 50 or 60 mM) did not induce germination of spores in buffer (at all concentrations tested only 0.1 log germination). Phase-contrast microscopy confirmed this finding, since only a very minor fraction of spores turned phase-dark in the presence of Ca^{2+}-DPA, even after overnight incubation. Also HP treatments of 200 MPa and 600 MPa for 15 min did not induce germination at 30 °C (0.0 +/- 0.4 log and 0.1 +/- 0.3 log germination respectively). Because there was no reduction of counts after HP, it can be concluded that no germination or inactivation took place due to the pressure treatment. Also in this case, phase-contrast microscopy confirmed the absence of germinated spores after the HP treatment. While the DPA release of 200 MPa treated spores (1.1 % +/- 0.3 %) showed no significant difference with spores incubated at P_{atm} (data not shown), spores subjected to 600 MPa released 8.4 +/- 2.1 % of the total DPA, an amount that was statistically significant ($p < 0.05$).

Efficient germination occurred in response to the gerMix (2.0 +/- 0.5 log CFU/ml) and due to the surfactant dodecylamine (4.5 +/- 0.3 log CFU/ml), based on the criterium of heat sensitivity. Plating of control samples indicated that neither the gerMix nor dodecylamine caused inactivation of the germinated spores within the course of the germination experiment. DPA release of spores in gerMix and dodecylamine, was 87.7 +/- 10.0 % and 66.1 +/- 5.0 % respectively (Fig. 6.1).

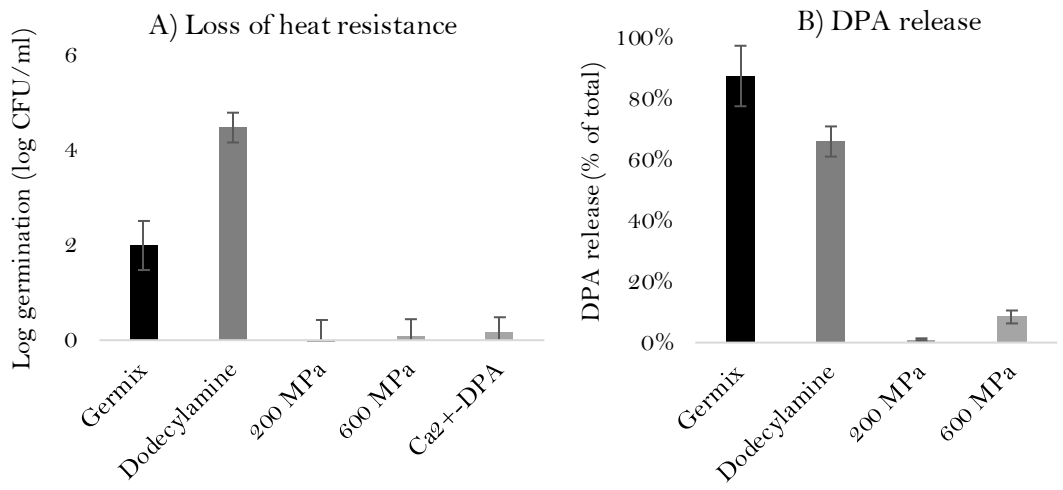

Fig 6.1: Spore germination by different potential inducers. Mean percentages +/- standard deviations are shown of three experiments using independent spore crops. **A)** Germination assessed by loss of heat resistance (65 °C/10 min), after incubation of heat activated spores for 1 h at 30 °C in the gerMix, in dodecylamine (3 mM) or in Ca^{2+}-DPA (60 mM), or after pressure treatment (200 / 600 MPa, 15 min, 30 °C) followed by an additional 4 h incubation at 30°C. Also 20, 30 and 50 mM Ca^{2+}-DPA were tested but showed no germination induction. **B)** DPA release of spores germinated by the same treatments as in (A), except Ca^{2+}-DPA. Release was measured after 4 h at 30 °C and is expressed relative to the total DPA release after treatment for 30 min at 98°C.

Since dodecylamine is a strong surfactant that has bactericidal properties, we also analysed the loss of heat resistance after washing away the compound after 4 h, and this reduced the amount of spores that are inactivated by heat to 0.9 +/- 0.2 log CFU/ml (not shown in Fig. 6.1). This suggested that the heat sensitivity of (most or all of) the spores in the presence of dodecylamine was not the consequence of genuine spore germination, but of direct spore inactivation by dodecylamine facilitated by heat. Also the DPA release of spores exposed to dodecylamine increased from 66 % before the heat treatment to 91.6 +/- 2.4 % after the heat treatment (the latter is not shown in Fig. 6.1). This is in agreement with earlier observations in *B. subtilis* where DPA release in the presence of dodecylamine also increased with increasing temperature [144]. Finally, despite the DPA release, the vast majority of spores remained phase-bright in the presence of dodecylamine, even after a heat treatment. This is in contrast to spores incubated in the gerMix, as shown in Fig 6.2, and the results therefore confirm that dodecylamine probably does not induce a physiological spore germination process.

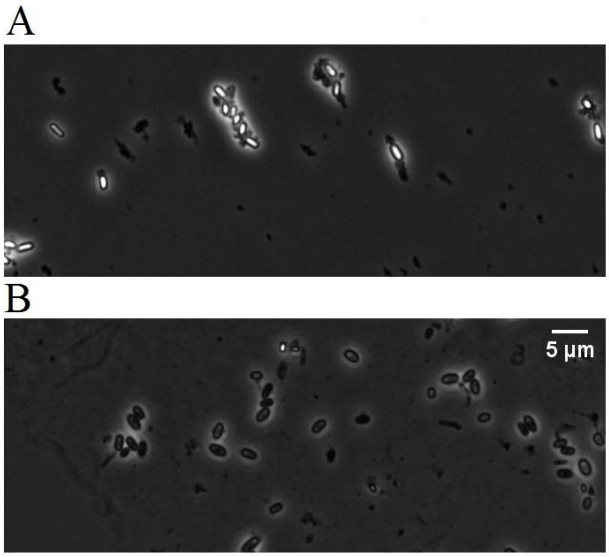

Fig. 6.2: Phase-contrast microscopy images of spores treated with germinants. The spores were incubated for 4 h at 30 °C in A) dodecylamine and B) the gerMix, whereafter a heat treatment was performed (65 °C/10 min) because it was shown that higher temperatures enhance dodecylamine's mode of action. Six microscopic fields were assessed for each treatment (with around 200 spores per field), and a representative image is shown.

After having identified effective germinants for *C. botulinum* NCTC 11219, we analysed four natural substances for inhibition of these germinants. Although it follows from our previous observations that dodecylamine does not induce genuine germination, we included this compound in the analysis because it remains of interest to understand how dodecylamine induces DPA release and whether this effect could be suppressed by the compounds that were tested on nutrient germination.

6.3.2 MIC on vegetative cells

The MIC of carvacrol, cinnamaldehyde, carrot seed EO and hop β-acids for vegetative cells of *C. botulinum* NCTC 11219 Δ*bont* was determined by the broth dilution assay to be 2 mM (308 ppm), 4 mM (503 ppm), 0.5 v/v % (5000 ppm) and 0.00025 v/v % (2.5 ppm) respectively. Therefore, their inhibiting action on germination was further assessed with concentrations around their MIC on vegetative cells.

6.3.3 Inhibition of nutrient induced germination

Spore germination in the gerMix was analysed in the presence of different concentrations of the compounds (Fig. 6.3). Carvacrol and cinnamaldehyde both inhibited germination, with 0.05 mM (8 ppm) carvacrol and 0.25 mM (31 ppm) cinnamaldehyde being the lowest tested concentrations that have a significant effect ($p < 0.05$).

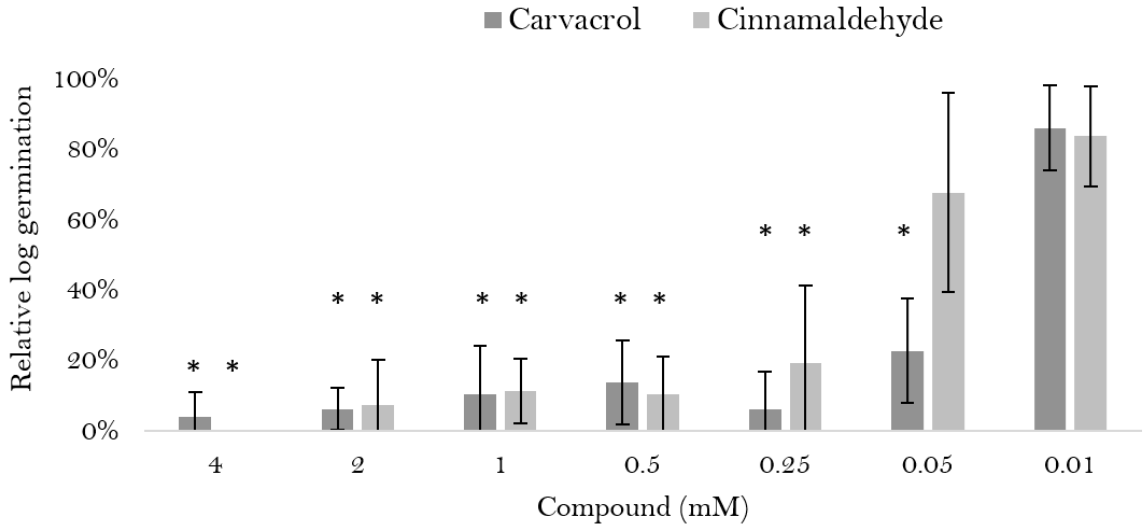

Fig. 6.3: Germination in gerMix with carvacrol or cinnamaldehyde (4 mM-0.01 mM) relative to germination in gerMix alone. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant differences ($p < 0.05$) in comparison to the control in gerMix alone are indicated with *.

Fig. 6.4 shows that carrot seed EO significantly inhibits nutrient germination at concentrations ≥ 0.005 v/v % (50 ppm), whereas hop β-acids do not inhibit germination at concentrations around the MIC for vegetative cells (0.00025 %). The inhibition of spore germination of NCTC 11219 by carrot seed EO contrasts with previous findings in our research group that this EO does not inhibit *B. cereus* spore germination [225]. Control experiments in which spores were incubated with the natural compounds in the absence of gerMix showed no germination nor inactivation (data not shown).

Phase-contrast microscopy allowed to conclude that inhibition by carvacrol, cinnamaldehyde and carrot seed EO occurs early in the germination process, since spores remained phase-bright when these compounds were added to the gerMix, even after 4 h incubation (Fig. 6.5). In contrast, hop β-acids did not prevent the spores from turing phase dark, confirming the lack of germination inhibition at concentrations around the MIC, as was also shown in Fig. 6.4. In addition, DPA release was significantly reduced by carvacrol, cinnamaldehyde or carrot seed EO (Fig. 6.6). Remarkably, a significant reduced release was also seen in the presence of hop β-acids, although the effect was smaller than for the others compounds. Controls were

also included where spores were incubated with the compounds without gerMix, showing no DPA release.

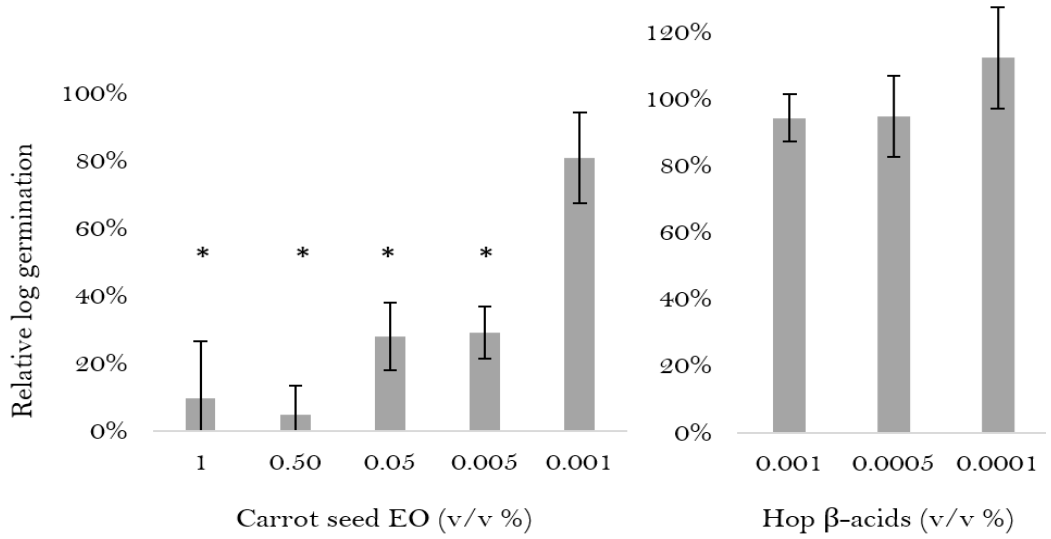

Fig. 6.4: Spore germination in gerMix in the presence of carrot seed EO (1 %-0.001 %) or hop β-acids (0.001 %-0.0001 %) relative to germination in gerMix alone. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant differences ($p < 0.05$) in comparison to the control in gerMix alone are indicated with *.

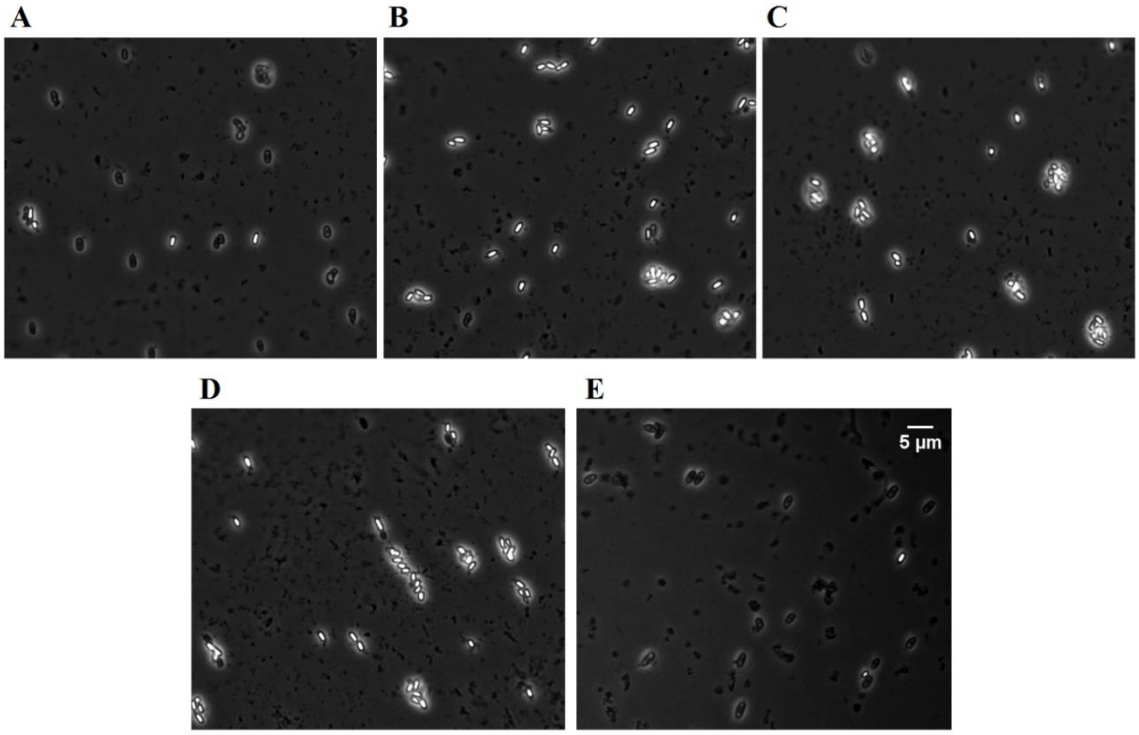

Fig. 6.5: Phase-contrast microscopy of spores incubated at 30 °C in gerMix with/without addition of natural compounds. Heat activated spores were resuspended in A) gerMix, B) gerMix with 4 mM carvacrol, C) gerMix with 4 mM cinnamaldehyde, D) gerMix with 1 % carrot seed EO, E) gerMix with 0.001 % hop β-acids. After 4 h, the spores were placed on an agarose pad for visualization. Six fields were assessed per condition (with around 200 spores per field), and a representative image is shown.

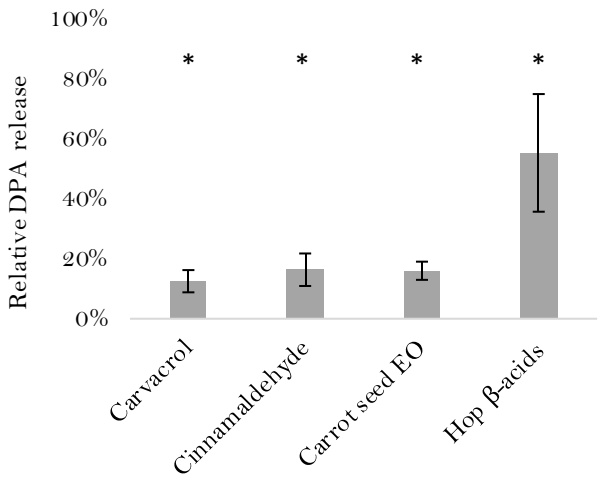

Fig. 6.6: DPA release of spores that were incubated for 4 h at 30 °C in gerMix with natural compounds, relative to the DPA release in gerMix alone. Heat activated spores were resuspended in gerMix, gerMix with 4 mM carvacrol, gerMix with 4 mM cinnaldehyde, gerMix with 1 % carrot seed EO and gerMix with 0.001 % hop β-acids. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant differences ($p < 0.05$) in comparison with the control in gerMix alone, are indicated with *.

Hop β-acids showed no inhibiting effect on heat sensitivity nor on the conversion from phase-bright to phase-dark, yet they did significantly reduce DPA release. We thus report the inhibitory effect of carvacrol, cinnamaldehyde and carrot seed EO on nutrient induced germination of NCTC 11219 Δ*bont* spores. For these compounds, the lowest concentration that had a significant effect on germination was much lower than the MIC for vegetative cells (Table 6.1).

Table 6.1: Minimal inhibitory concentration (MIC) of four natural compounds on vegetative cells and on nutrient induced spore germination of NCTC 11219 Δ*bont*.

Compound	**MIC on vegetative cells**	**MIC on nutrient germination ***
Carvacrol	2 mM / 308 ppm	0.05 mM / 8 ppm
Cinnamaldehyde	4 mM / 503 ppm	0.25 mM / 31 ppm
Carrot seed EO	0.5 v/v % / 5000 ppm	0.005 v/v % / 50 ppm
Hop β-acids	0.00025 v/v % / 2.5 ppm	No inhibition shown

* The MIC on vegetative cells is determined after 24 h by the broth dilution method, as the lowest concentration that inhibits vegetative growth, whereas the MIC on nutrient germination is determined after 1 h as the lowest concentration that has a significant effect on nutrient induced germination.

The MIC on nutrient induced germination of carvacrol, cinnamaldehyde and carrot seed EO was respectively 40-, 16- and 100-fold lower than the MIC on vegetative cells. The observation for carrot seed EO is of particular interest since it has been reported previously by our group that this EO could not inhibit germination of *B. cereus*, while ten other EOs that were tested did have an effect [225]. It is still unclear how these natural compounds inhibit germination and it would be interesting to investigate this further. Additionally, the bioactive compounds in carrot seed EO should be further identified. The oil that is used here consists mainly of carotol (66 %) and carotenol (16 %). An antifungal effect has already been described for carotol [217], so it would be interesting to use the pure compound in future testing. Finally, while the MIC of hop β-acids on vegetative cells is particularly low, no inhibition on germination was observed around this concentration range. One possibility could be that the hop β-acids are too large molecules to insert or pass the cortex/coat layers. Moreover, it could be that higher concentrations are required for an inhibitory effect. Nevertheless, these β-acids can be of major importance for the food industry because of their low MIC on vegetative cells.

6.3.4 Inhibition of dodecylamine induced germination

The same natural compounds that were tested for inhibition of nutrient induced germination, were tested on the apparent germination induced by dodecylamine. First the effect of the compounds on the induction of heat sensitivity was assessed. The results for carvacrol and cinnamaldehyde are shown in Fig. 6.7 and the results for carrot seed EO and hop β-acids are shown in Fig. 6.8. Both cinnamaldehyde and carrot seed EO showed an inhibitory effect in a concentration dependent manner, while carvacrol and hop β-acids showed no effect at the tested concentrations. The lowest concentrations that had a significant effect were 0.5 mM cinnamaldehyde and 0.5 % (v/.v) carrot seed EO ($p < 0.05$).

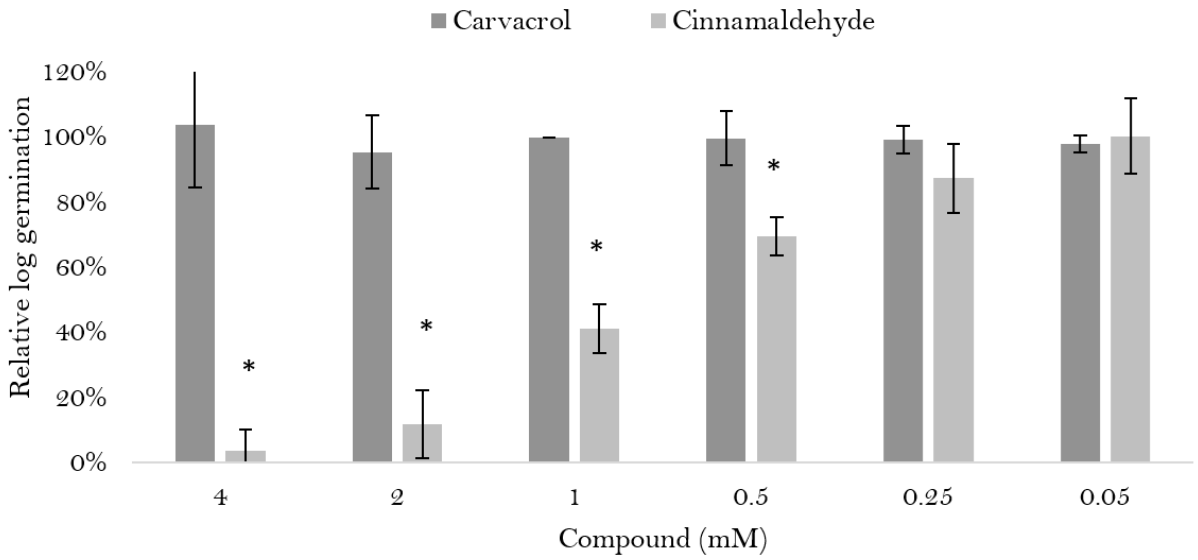

Fig. 6.7: Apparent spore germination in the presence of dodecylamine with carvacrol or cinnamaldehyde (4 mM-0.05 mM) relative to apparent germination in the presence of dodecylamine without the compound. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant differences ($p < 0.05$) in comparison to the control in dodecylamine alone are indicated with *.

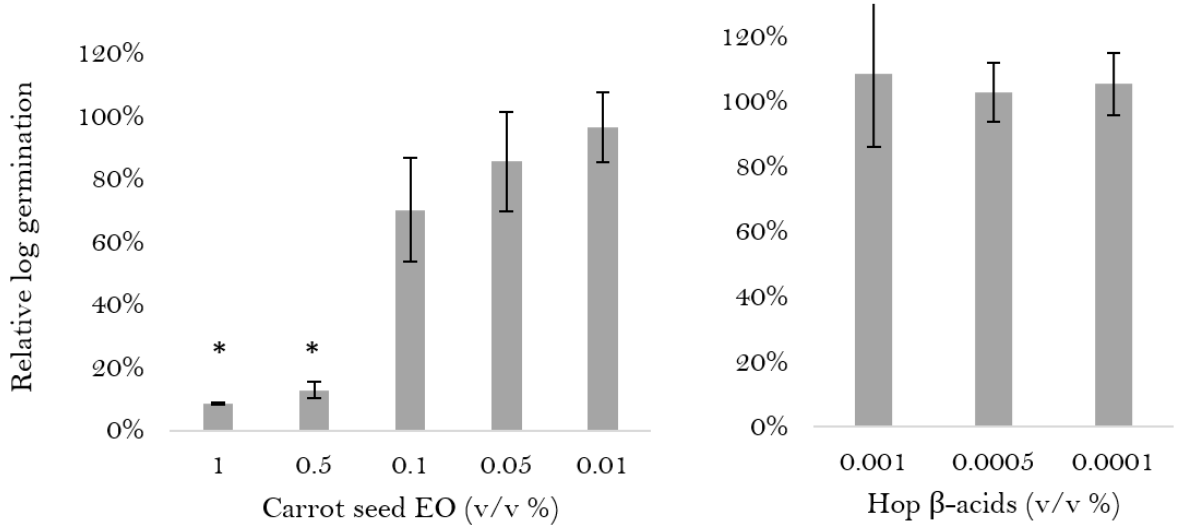

Fig. 6.8: Apparent spore germination in the presence of dodecylamine with carrot seed EO (1 %-0.01 %) or hop β-acids (0.001 %-0.0001 %) relative to apparent germination in the presence of dodecylamine without the compound. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant differences ($p < 0.05$) in comparison to the control in dodecylamine alone are indicated with *.

Furthermore, DPA release induced by dodecylamine was tested with the addition of each of the four natural compounds (Fig. 6.9). DPA measurements showed a significant reduction in DPA release in dodecylamine with the addition of cinnamaldehyde compared to release of spores in the presence of dodecylamine alone. It remains unclear why no reduction in DPA release was shown in the presence of carrot seed EO, while a significant effect on loss of heat resistance was observed (at concentrations ≥ 0.5 %). The other compounds showed no effect

on DPA release due to dodecylamine. Spores only incubated with the compounds (without dodecylamine), showed no DPA release.

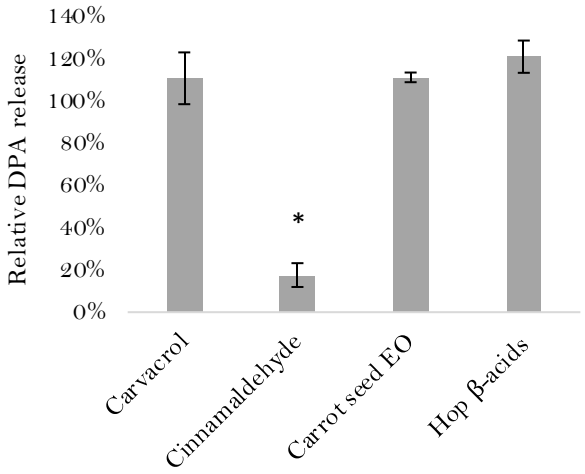

Fig. 6.9: DPA release of spores that were incubated for 4 h at 30 °C in dodecylamine with addition of natural compounds, relative to the DPA release in dodecylamine alone. Spores were resuspended in 3 mM dodecylamine with 4 mM carvacrol, 4 mM cinnamaldehyde, 1 % carrot seed EO or 0.001 % hop β-acids. Mean percentages +/- standard deviations of triplicate experiments using independent spore crops are shown. Significant reductions ($p < 0.05$) in comparison with the control in dodecylamine alone, are indicated with *.

Since previous observations led us to conclude that dodecylamine does not induce genuine germination, we speculated that the effect of cinnamaldehyde could be due to a reaction of its electrophilic α,β unsaturated aldehyde group with the primary amino group of the surfactant. When cinnamylalcohol, a structural analogue of cinnamaldehyde in which the aldehyde group is replaced by an alcohol group was used instead of cinnamaldehyde, no inhibition of dodecylamine induced apparent germination was observed (data not shown). This observation is in agreement with our hypothesis (although it is not evidence for a chemical reaction), and may suggest that the amine group of dodecylamine plays an important role in its effect on spores. The latter is in accordance with a previous suggestion that the NH_2 head group is capable of forming salt bridges with carboxylate anions in the spore cortex, causing the core to take up small amounts of water [145].

6.4 Conclusion

In this chapter, different germination inducers were tested on spores of strain NCTC 11219 Δ*bont* (Fig. 6.1). Exogenous Ca^{2+}-DPA (up to 60 mM) was not able to induce germination. In *B. subtilis*, similar concentrations of Ca^{2+}-DPA trigger germination by activating the cortex hydrolase CwlJ, and since gIICb strains do not carry a CwlJ homolog, the failure of Ca^{2+}-DPA to induce germination may seem evident. However, Ca^{2+}-DPA germination is not always clearly linked to CwlJ, since *C. difficile* spores do not germinate with Ca^{2+}-DPA although the organism encodes a CwlJ homolog (30 % amino acid identity with *B. subtilis* CwlJ) [143], while *C. perfringens* spores germinate in response to Ca^{2+}-DPA despite the absence of a *cwlJ* homolog in the genome. In addition, HP treatments (200/600 MPa, 15 min, 30 °C) also failed to induce germination based on loss of heat resistance, and only the 600 MPa treatment induced a small amount of DPA release. This is in accordance with previous reports that showed that HP cannot induce gIICb spore germination efficiently at moderate temperatures, and with the more general observation that spores of clostridia tend to germinate only weakly or not at all following HP treatment [152,160,228,229].

On the contrary, the nutrient mixture L-alanine/L-lactate/$NaHCO_3$ induced very efficient germination whereas the non-nutrient dodecylamine only showed to induce apparent germination, based on the heat sensitivity criterium and DPA release. Therefore, the ability of four natural antimicrobial compounds to inhibit germination by these two inducers was subsequently tested. Although inhibition of nutrient induced germination is most relevant for spore control in foods, inhibition of dodecylamine induced germination is of interest as well since this inducer follows a different mechanism that is less understood. We showed that carvacrol, cinnamaldehyde and carrot seed EO can inhibit nutrient germination of *C. botulinum* spores at concentrations many times lower than the MIC (Table 6.1). This offers an advantage for the food industry since these compounds have quite low sensory thresholds, making application in foods sometimes difficult [230,231]. Further, carrot seed EO and cinnamaldehyde were capable of reducing dodecylamine's effect on heat resistance, although for carrot seed no reduction in DPA release was shown. While cinnamaldehyde's inhibitory effect could maybe be explained by a reaction between the electrophilic α,β unsaturated aldehyde group and the cationic amine group of dodecylamine, the mechanism of inhibition by carrot seed EO remains unclear.

Chapter 7*

Canonical germinant receptor is dispensable for spore germination by nutrient or non-nutrient germinants in *C. botulinum* group II strain NCTC 11219

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* This chapter is based on a manuscript submitted for publication: Clauwers, C., Lood, C., van Noort, V. & Michiels, C. Canonical germinant receptor is dispensable for spore germination in Clostridium botulinum group II strain NCTC 11219.

C. Lood and V. Van Noort contributed by conducting bioinformatic analysis of genome sequences.

7.1 Introduction

In the natural environment, spore germination is induced when specific nutrients bind to spore germinant receptors (GRs) in the spore membrane, thus signalling that conditions are favorable for outgrowth (see 2.2.1). This initiates a self-propagating and irreversible cascade of events that does not require metabolic energy, starting with the release of monovalent cations H^+, Na^+, K^+ and Ca^{2+}-DPA from the spore core, followed by degradation of the spore cortex and core rehydration, eventually leading to reinitation of metabolism and outgrowth. Core rehydration is a key event in this process that underlies both the loss of the spores' resistance properties and the activation of metabolism [119,127,232].

The nutrients that trigger germination are species and strain specific and most commonly include one or more L-amino acids, purine ribosides and D-sugars [118]. GRs designated as Ger receptors have been first identified in *B. subtilis* and later shown to be conserved in spore-forming bacilli and clostridia, with few exceptions. However, the diversity among Ger receptors is large. In *B. cereus*, where this has been studied in most detail, a recent study defined eleven different phylogenetic clusters of Ger receptors, with the number of receptors per strain varying from four to ten [233]. The number and types of Ger receptors present in a strain are believed to reflect the spectrum of germinants that it responds to. In *B. subtilis* strain 168, for example, GerA responds to D-alanine, while GerB and GerK cooperatively respond to a germinant mixture of D-alanine, glutamate, fructose and K^+. However, the link between the germination response and the presence of specific Ger receptors is usually more complex, as exemplified by the study of Warda et al. (2017), who failed to establish such a link in seventeen *B. cereus* strains with a variable Ger receptor content [233]. This may be due to various reasons, including the existence of a functional hierarchy and interactions between different receptors, and the fact that receptors may respond to more than one germinant and that their response may be influenced by so-called co-germinants [234]. Knockout analysis has also indicated that Ger receptors of the same type may have a different contribution to the germinant response in different bacteria. For example, GerL was linked to L-alanine germination in *B. cereus* 569, but did not affect L-alanine germination in *B. cereus* ATCC 14579 [16,235]. Individual knockout of all *ger* operons in *B. cereus* ATCC 14579 failed to identify a role in germination for three (GerK, GerL and GerS) out of the seven Ger receptors, although all operons were transcribed during sporulation [16]. A similar finding was reported in *B. megaterium* PV361, where only GerK seems to play a major role in germination, while the other four Ger receptors have only minor effects [236]. Clarification of the structure-function relationship of Ger receptors has also been hindered by the difficulty to isolate functional receptors, because they are membrane-

associated complexes composed of three subunits and associated with other proteins in a so-called germinosome complex [237,238].

Ger-type GRs are typically composed of three protein subunits designated A, B and C, that are usually encoded in a tricistronic operon. The A protein comprises four to eight predicted membrane-spanning domains, as well as large N- and C-terminal hydrophilic domains. The B protein contains seven to twelve transmembrane helices, and is structurally related to a superfamily of membrane-associated single-component membrane transporters, although the sequence similarity is low. The C subunit of the Ger receptor is predicted to be a lipoprotein that is anchored to the outer surface of the spore inner membrane [90,119,127]. Christie et al. (2008) could alter the germinant response of *B. megaterium* spores by replacing the B subunit of a receptor by that of another strain, thus suggesting that this subunit is responsible for germinant binding [239]. Remarkably, while the available evidence indicates that all subunits are required to form a functional receptor in bacilli, several clostridia, like *C. beijerincki*, *C. butyricum*, *C. asparagiforme*, encode only a single A subunit, and it is unclear whether and how this subunit is involved in spore germination in these bacteria [127].

Brunt *et al.* (2016) recently reported on the diversity of Ger gene clusters in groups I-IV of *C. botulinum*, based on *in silico* analysis of 148 *C. botulinum* and 8 *C. sporogenes* genomes [120]. The latter were included because of their close relatedness to gICb. Four different *ger* clusters were identified (*gerX1-4*), which were further divided into subtypes designated with an additional letter (see Fig. 2.3). gICb and *C. sporogenes* strains typically contain three to five different Ger receptors, encoded by *gerX1a/c/d*, *gerX2a/b/c* and/or *gerX3a*. In general, spores from these strains germinate in response to various amino acids in combination with L-lactate, although the latter is not always essential [64,121]. The functionality of *C. botulinum* Ger receptors has been experimentally studied in gICb strain ATCC 3502, which has a *gerX1a*, *gerX1d* and *gerX2b* cluster, and in *C. sporogenes* ATCC 15579, which has a *gerX1a*, *gerX1d*, *gerX2c and gerX3a* cluster [64]. Construction and analysis of insertional knockouts in the A subunit encoding gene of each individual *ger* cluster revealed that both *gerX1a* and *gerX1d* were essential for amino acid germination in the gICb strain, while *gerX2b* was completely dispensable. In the *C. sporogenes* strain, in contrast, only *gerX1d* was essential for germination, while *gerX1a* was dispensable, and *gerX2c* and *gerX3a* influenced the rate, but not the extent of germination. No indications were found in regard to the germinant specificity of the receptors, since the effect of knocking out a receptor was always the same, irrespective of the germinant used (L-ala, L-cys, L-met, L-ser, L-phe; for all together with L-lactate). In *C. sporogenes*, four triple knockout mutants, each carrying only one intact *ger* cluster, were also constructed. Only the mutant

with an intact *gerX1d* cluster retained wild type germinant responsiveness, while all other mutants failed to germinate. While this study yielded important insights in Ger receptor function, it also has an important limitation because the insertional knockout of the A subunit gene in a *ger* cluster does not necessarily abolish expression of the B and C subunits. This is certainly the case for the *gerX3a* cluster, which has a bicistronic organization, with *gerB* transcribed in the opposite direction as *gerA* and *gerC*.

Much like gICb, gIICb spores germinate in response to several amino acids in combination with L-lactate, but other than in gICb, L-lactate seems to be essential for germination. In a systematic study with three gIICb strains, L-alanine, L-cysteine and L-serine were the amino acids that induced the strongest germination response [122]. In addition, it was already reported earlier that gIICb spores germinated in response to amino acids at pH 9 in absence of lactate, as well as the combination of L-alanine with glucose, galactose or maltose at neutral pH [123]. As opposed to gICb, analysis of gIICb genome sequences indicates that they produce only one Ger receptor, encoded by a *gerX3b* type cluster [120], but the function of this receptor remains to be investigated.

Mutational studies have also been conducted in *C. perfringens*, which contains a *gerX3* like locus designated *gerK*, and a distantly located monocistronic *gerAA* gene [124,125]. It was concluded that GerKA, GerAA and GerKB only play auxiliary roles since inactivation of either of these proteins had no significant effect on germination. GerKC, on the other hand, was required for the response to germinants such as KCl, L-asparagine, or a L-asparagine–KCl mixture. The finding that the A and B subunits are dispensable in *C. perfringens* is in striking contrast with the situation in *B. subtilis* [240].

Despite the wide distribution of Ger-type receptors in sporulating bacteria, there is evidence for the existence of other receptor types. Although the genome sequences of at least two *Clostridium* species, *C. bartletti and C. difficile*, do not contain Ger gene homologs, spore germination in these bacteria is also induced by specific germinants, consistent with a mechanism involving one or more specific receptors. *C. difficile* spores do not respond to amino acid germinants, but to taurocholate, a characteristic component of bile, which allows the spores to recognize their primary niche, the animal gut. Francis et al. (2013) found that several *C. difficile* germination-null mutants had mutations in *cspC*, which encodes a catalytically inactive homolog of the germination protease CspC [126]. Interestingly, a mutant that germinated in response to chenodeoxycholic acid, which normally is a competitive inhibitor of germination, was also found, and this led the authors to propose that CspC acts as the GR

for taurocholate in *C. difficile* [126]. Upon germinant binding, CspC is postulated to undergo a conformational change that is transmitted to CspB, which would then activate the cortex hydrolase SleC by proteolytic cleavage of its pro-region. Several other clostridia, including gIICb strains, contain Csp related serine proteases, but their possible role as GRs has not yet been studied [127].

It is clear from the above that the mechanisms of spore germination can vary substantially, and that in clostridia in particular, the role of the Ger receptors and the possible existence of additional GRs requires further investigation. In gIICb strains, specifically, the presence of only a single Ger-type receptor contrasts with the large variety of amino acids that can trigger germination. It seems unlikely that GerX3b can act as a specific receptor for all these germinants. In the present work, we deleted the entire *gerBAC* locus encoding the GerX3b in gIICb strain NCTC 11219 to analyse its role in germination with different nutrients and the non-nutrient dodecylamine. The deletion was accomplished with the use of a *pyrE*-based gene replacement method that we used previously to remove the botulinum neurotoxin gene from the same strain (Chapter 4).

7.2 Materials and Methods

7.2.1 Bacterial strains and growth conditions

This work was conducted in an atoxigenic Δ*bont*::*ermB* and uracil auxotrophic Δ*pyrE* mutant of strain NCTC 11219 (constructed in Chapter 4), further in this chapter shortly named Δ*bont* Δ*pyr*. This strain was used for biosafety reasons, and because of the possibility to use *pyrE in trans* as a negative selection marker for replacement of the *gerBAC* locus. Clostridial cultures were routinely grown at 30 °C in TPGY broth and plated on solid RCM or TPGY agar, whereas TYG agar was used in mating (see 4.2.1). Vegetative cultures were manipulated and incubated in a Don Whitley DG250 anaerobic workstation using overnight pre-reduced media. Spores suspensions were handled in open air, and transferred to the workstation only for experiments involving outgrowth. Production and purification of spore crops was done as described in 4.2.2.

E. coli strains were grown in LB or on LB agar at 37 °C. *E. coli* DH5α was used for cloning and maintenance of plasmids, while *E. coli* CA434 (HB101 containing plasmid R702,[185]) was used as conjugation donor. Media were supplemented with the following antibiotics (Applichem): thiamphenicol (Tm, 15 µg/ml in agar, 7.5 µg/ml in broth) and spectinomycin

(Sp, 600 µg/ml) for *C. botulinum*, cycloserine (Cy, 250 µg/ml), chloramphenicol (Cm, 25 µg/ml in agar, 12.5 µg/ml in broth) and Sp (100 µg/ml) for *E. coli*. 5-Fluoroorotic acid (5-FOA, 500 µg/ml (Manchester Organics) was used in screening for loss of the *pyrE*-expressing plasmid pMTL84151Δ*gerBAC*.

7.2.2 Plasmid construction

The plasmid pMTL84151Δ*gerBAC* was constructed to replace the *gerBAC* locus with the Sp resistance marker *aad9* in strain Δ*bont* Δ*pyr*. All primers used are listed in Table 7.1 and were obtained from Integrated DNA Technologies. First, *pyrE* (675 bp) was amplified from start to stop codon with primers pyrE11219_F and pyrE11219_R, restricted with NdeI/SacI and placed after the p_{fdx} promotor in pMTL83353, opened with the same enzymes. Hereafter, the fragment containing p_{fdx} and *pyrE* was amplified with primers pMTL83353_F and pyrE11219_R, digested with SbfI and SacI and cloned in pMTL84151, restricted with the same enzymes. Flanking loci of *gerBAC* (5' locus: 932 bp, 3' locus: 1091) were amplified from gDNA of NCTC 11219 using primer pairs ger5'F/ger5'R and ger3'F/ger3'R, respectively. The *aad9* locus (1009 bp) was amplified from plasmid pMTL83353 with primers aad9_F and aad9_R. The amplified 5' and 3' loci, the *aad9* fragment and pMTL84151 containing p_{fdx} *pyrE*, opened by PCR using primer pair pMTL84151_openF / pMTL84151_openR, were cloned together using Gibson assembly, following the suppliers protocol (New England Biolabs, Hitchin, UK). The plasmid construct designated pMTL84151Δ*gerBAC* was verified by PCR and sequence analysis, and transferred to *E. coli* CA434 by electroporation.

Table 7.2: Oligonucleotides used for cloning and construct verification. Restriction sites are underlined: NdeI (CATATG), SacI (GAGCTC) and SbfI (CCTGCAGG). The small letters indicate the overhang region of the primer, necessary for annealing fragments via Gibson assembly.

Name	Sequence (5'-3')
pyrE11219_F	AGGCATATGGAAGCATATAAAAAAGAG
pyrE11219_R	CTTGAGCTCCTACTTAGCACCATATTC
pMTL83353_F	GAGCCTGCAGGATAAAAAAATTGTAG
pMTL84151_openR	ttctggtgatttaactttagCTCCTACTTAGCACCATATTC
pMTL84151_openF	ccgtcgttttacaacgtc
ger5'F	gatgaaattaaaactagaatagatgaatattacaaagaatatggtgctaagtaggagCTAAAGTTAAATCACCAGAAGG
ger5'R	GCACTTTACTTATACATATATCACTAATGAC
ger3'F	ATGAAGGTATAATTTTAAAGATGCTCTAAAATCTC
ger3'R	acgacgttgtaaaacgacggCTAAACATTTCTCTACATCTGC
aad9_F	tctttattttagtcattagtgatatatgtataagtaaagtgcCAATGAATAGGTTTACACTTACTTTAGTT
aad9_R	aataacagagattttagagcatctttaaaattataccttcatAATAAAACAAAAAAATTGAAAAAAGTGTTTCCA CCA
ΔgerBAC_upF	GTTATAGCATGTAAATCAACCACGC
ΔgerBAC_downR	TCTTAGCTCCATTAATTTCAGCAC

7.2.3 Construction of the Δ*gerBAC* deletion mutant

Plasmid pMTL84151Δ*gerBAC* was introduced into NCTC 11219 Δ*bont* Δ*pyr* by conjugation (see 4.2.4), using selection on RCM agar with Tm and Cy. Purified transconjugants were resistant to Sp and sensitive to 5-FOA, confirming expression of the plasmid *aad9* resistance marker and *pyrE* gene, respectively.

Transconjugants were serially grown in TPGY with Sp, and every round was then plated on TPGY with Sp and 5-FOA to select for clones in which double homologous recombination with the flanking loci of *gerBAC* as well as loss of the plasmid had occurred. Multiple attempts of plating were needed before resistant colonies appeared. Then, up to 30 colonies could be selected out of 200 µl of an overnight culture (i.e. about 10^7 cells), and plasmid loss was confirmed by the loss of Tm resistance of the clones. PCR and sequence analysis with primers ΔgerBAC_upF / ΔgerBAC_downR, annealing on the chromosome outside the homologous fragments, were performed to confirm that *gerBAC* was deleted and replaced by *aad9*. The mutant was designated NCTC 11219 Δ*bont* Δ*pyr* Δ*gerBAC*::*aad9* (in this manuscript further described as Δ*bont* Δ*pyr* Δ*gerBAC*).

7.2.4 Whole genome sequencing

To further confirm the absence of the *gerBAC* genes, the Δ*bont* Δ*pyr* Δ*gerBAC* strain and its parent Δ*bont* Δ*pyr* were subjected to WGS analysis on an Illumina MiSeq sequencer. First,

gDNA from both strains was isolated from overnight cultures using the GeneJET Genomic DNA purification kit (Thermo Scientific). DNA purity and concentration was assessed by Nanodrop analysis, gel electrophoresis and Qubit (Thermo Scientific) analysis. Paired-end libraries were constructed using the NEBNext Ultra gDNA library prep protocol with an average insert size of 240 bp, and analysed on the Agilent BioAnalyzer (VIB nucleomics core) resulting in on average 1.2 million reads per sample. Reads were analysed with Qiagen's CLC Genomics Workbench version 8.5 (http://www.clcbio.com/), and included standard quality control, read trimming and filtering (reads < 15 nucleotides were discarded, quality score limit = 0.01, ambiguous nucleotides trim limit = 2), and read mapping to *C. botulinum* NCTC 11219 reference WGS with accession number JXMR00000000 (using parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8), yielding on average a 43-fold genome coverage for the parental strain and 51-fold coverage for the Δ*gerBAC* mutant.

7.2.5 Chemicals and stock solutions

Different germinant mixtures were prepared freshly before use in 100 mM Tris-HCl buffer pH 7.4, as twofold concentrated working solutions, from the following chemicals: L-alanine (Sigma), L-lactate sodium salt (Acros), $NaHCO_3$ (Acros), L-serine (Acros), L-cysteine (Acros), L-threonine (Acros), inosine (Sigma) and D-glucose (Acros). A 1 M stock solution of dodecylamine (Acros) was freshly prepared in ethanol, and further diluted in 100 mM Tris-HCl buffer to 6 mM (= twofold of working concentration).

7.2.6 Germination assays

Spore crops were centrifuged (3400 x g, 10 min, 4 °C), resuspended in 100 mM Tris-HCl buffer, and heated for 10 min at 65 °C to inactivate any residual vegetative cells and spores that would have spontaneously germinated during storage, and also to activate dormant spores for germination. Hereafter a sample was taken from the suspension, diluted in Tris-HCl buffer and plated on TPGY to determine the initial spore count N(t0). The remainder of the heat activated spore suspension was mixed with an equal volume ratio of a twofold concentrated germinant solution (or buffer as a negative control), and incubated for 4 h at 30 °C. The suspension was then heated for 10 min at 65 °C and plated on TPGY, to determine the counts N(t4-HT). Germination was then expressed using the following formula:

$$\textbf{Log germination (log CFU/ml)} = \log_{10}(\textbf{N(t0)} / \textbf{N(t4-HT)})$$

7.2.7 DPA measurements

After 4 h of incubation, spores were removed from the germinant solution by centrifugation (12 000 x g for 5 min), whereafter the supernatant was used for DPA analysis. Hundred µl of the supernatant was mixed with an equal volume of 20 mM $TbCl_3$ in a 96-well black microtiter plate (Greiner Bio-one) [227]. Fluoresence measurements were done in a spectrofluorometer (Synergy Mx-biotek) with excitation and emission wavelengths of 270 and 545 nm, respectively. DPA release was expressed relative to the total spore DPA content, which was measured after treatment of the spores at 98 °C for 30 min [144].

7.2.8 Microscopy

Spore were immobilized on thin pads of 1 % agarose in Tris-HCl buffer with/without addition of germinants. Microscopy pictures were taken with a Ti-Eclipse inverted microscope (Nikon) equipped with a CoolLED pE-100 camera. Images were acquired using NIS-Elements (Nikon) and resulting pictures were further handled with open source software ImageJ.

7.2.9 Statistical analysis

The statistical significance of the extent of germination and DPA release was performed using the Student's T-test with a significance level of 0.05.

7.2.10 Functional analysis of 152 gIICb strains

The raw Illumina reads of a diverse set of 152 gIICb strains that have been recently published [165] (although after Chapter 3 was published) were retrieved from the NCBI Sequence Read Archive database (Accession number: SRP059342, no corresponding assembly available). The fastq files were processed with BBduk for removal of adapter contamination, trimming (Phred score > 28), and size exclusion (read length > 50bp). Each fastq file was subsequently inspected with FastQC for quality control [241]. The genome of each strain was assembled with SPAdes [242], and the quality of the assembly assessed with QUAST [243]. Functional annotation was done using Prokka [244] with a custom protein database created from strains of the *Clostridium* genus. The protein content of each genome was finally queried using Blastp against the GerX3b present in gIICb strain Eklund-B17.

7.3 Results

7.3.1 Construction of the Δ*gerBAC* deletion mutant

The deletion of the *gerBAC* locus was done in strain NCTC 11219 Δ*bont* Δ*pyr* of which the construction was described in Chapter 4. Besides having the advantage of being nontoxigenic, the *pyrE* deletion renders this strain resistant to 5-FOA, making it possible to use the *pyrE* gene as a negative selection marker. Plasmid pMTL84151Δ*gerBAC* (Tm^RSp^R) was conjugated to this strain to allow replacement of *gerBAC* with *aad9*. Since *pyrE* is also expressed on the plasmid, it renders the strain 5-FOA-sensitive. Then, plating on RCM with 5-FOA and Sp selects for clones in which double homologous recombination as well as loss of the plasmid had occurred. PCR analysis confirmed that *gerBAC* was deleted and replaced by *aad9*. WGS analysis confirmed this, and additionally indicated that the *gerBAC* locus had not translocated elsewhere in the genome, but was completely absent in strain Δ*bont* Δ*pyr* Δ*gerBAC*.

The spore yield of the Δ*gerBAC* strain (6.9 +/- 0.6 log cfu/ml; n = 6) was unaffected ($p > 0.05$) compared to that of the parental Δ*bont* Δ*pyr* strain (7.2 +/- 0.4 log cfu/ml; n = 6). Colony formation from spores of the Δ*gerBAC* strain was not delayed and the colonies were indistinguishable from those of the parent strain. Spore counts, determined after a heat treatment, were stable for at least four months at 3 °C.

7.3.2 The role of GerX3b in germination induced by L-alanine/L-lactate/$NaHCO_3$

Since the combination of L-alanine with L-lactate and $NaHCO_3$ is a very efficient inducer of spore germination in a wide range of gIICb strains [122,123], we first used this germinant mixture to analyse the impact of deletion of the GerX3b receptor. Unexpectedly, germination of the mutant, as assessed by heat treatment and plating and by DPA release (1.5 +/- 0.6 log germination ; 69.3 +/- 10.7 % DPA release), was not significantly different from that of the parent strain (1.3 +/- 0.3 log germination ; 83.2 +/- 8.2 % DPA release) (Fig. 7.1). Replacement of L-alanine by D-alanine reduced germination of both strains to a background level comparable to that observed in the absence of germinants, indicating stereospecificity of the L-alanine response. Germination was also monitored by phase-contrast microscopy of spores deposited on agar pads. When the L-alanine/L-lactate/$NaHCO_3$ germinant mixture was incorporated in the agar, almost half of the spores had already turned phase-dark by the time the slide could be viewed, but there was no difference between both strains in the fraction of phase-dark spores or in their appearance (Fig. 7.1). When no germinants were included in the agar pads, the spores of both strains remained phase-bright.

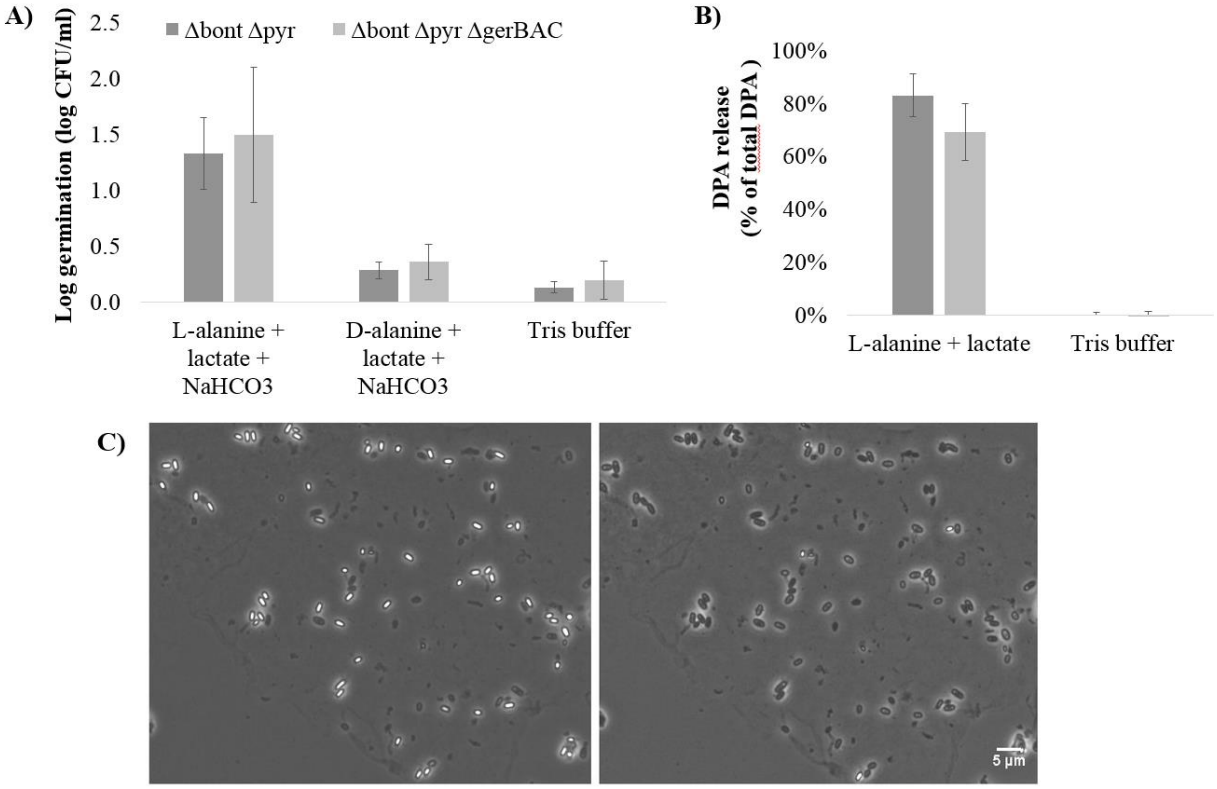

Fig. 7.1: Spore germination of Δ*gerBAC* spores compared to the parental strain, in L-alanine/L-lactate/$NaHCO_3$ (all at 50 mM) in 100 mM Tris-HCl buffer (pH 7.4). A) Germination assessed by loss of heat resistance (65 °C/10 min), after incubation of heat activated spores for 4 h at 30 °C in the germinant mixture. D-alanine (50 mM) was used to demonstrate the stereospecific action of germination induction in comparison to L-alanine. Means +/- standard deviations are shown of three experiments using independent spore crops. No significant differences were found ($p > 0.05$) between the two strains. B) DPA release of heat activated spores incubated for 4 h at 30 °C in the germinant mixture, relative to the total DPA content. Mean percentages +/- standard deviations are shown of three experiments using independent spore crops. No significant differences were found ($p > 0.05$) between the two strains. C) Spores of the Δ*gerBAC* mutant visualized with phase-contrast microscopy 5 min (left) and 1 h (right) after deposition on an agar pad containing L-alanine/L-lactate/$NaHCO_3$ germinant mixture. A considerable fraction of spores had already become phase-dark after 5 min, and spore germination was > 90 % after 1 h. A similar result was obtained for the parental strain (picture not shown).

7.3.3 The role of GerX3b in germination induced by other nutrient germinants

Because deletion of the GerX3 receptor showed no effect on germination induced by L-alanine/L-lactate/$NaHCO_3$, we further tested other nutrient mixtures previously described to germinate gIICb spores (listed in Table 7.2) [122,123]. All mixtures were first tested on the parental strain and mixtures which induced at least a 0.4 log germination were subsequently also used for the Δ*gerBAC* mutant. Besides L-alanine, both L-serine and L-cysteine in combination with L-lactate and $NaHCO_3$ have been previously reported as very efficient inducers of germination of gIICb spores. This was confirmed for the spores of our strain, and we additionally observed very efficient germination in L-threonine/L-lactate/$NaHCO_3$, as well as the combination inosine/L-alanine/$NaHCO_3$, two mixtures previously reported as

moderate germination inducers [122,123]. Unexpectedly, no statistical differences were found in spore germination with any of these germinants between the parental strain and the Δ*gerBAC* mutant ($p > 0.05$).

No germination was obtained with glucose/L-alanine/$NaHCO_3$, glycine, and glycine/L-lactate/$NaHCO_3$, although these were previously reported to induce germination (Table 7.2). Ando (1971) reported gIICb spore germination by the single amino acids, L-alanine, L-cysteine, L-serine at pH 9.0 in the presence of $NaHCO_3$ [123]. However, we were not able to test these mixtures by assessing loss of heat resistance, because Δ*bont* Δ*pyr* spores (as well as Δ*bont* spores) already became heat sensitive in carbonate buffer (pH 9.0) without addition of nutrients. Because the fraction of phase-dark spores remained negligible, even after overnight incubation in the germinant mix, we concluded that these amino acids at pH 9.0 do not induce germination in the NCTC 11219 strain.

7.3.4 The role of GerX3b in dodecylamine induced apparent germination

Because loss of the putative GerBAC receptor did not affect spore germination by nutrient germinants, we next evaluated the effect on apparent spore germination by the non-nutrient germinant dodecylamine. Spores in the presence of dodecylamine (3 mM, 4 h at 30 °C) showed strongly reduced counts after heat treatment, and there was again no significant difference ($P > 0.05$) between the parental strain and the Δ*gerBAC* mutant (Table 7.2). In addition, DPA release induced by dodecylamine treatment was not different between the Δ*gerBAC* mutant (42.4 +/- 3.2 %) and the parental strain (39.1 +/- 9.6 %) ($p > 0.05$). Similar low values of DPA release have been reported previously for dodecylamine induced germination at relatively low temperatures, and higher temperatures stimulated DPA release [34]. Furthermore, as was also shown in Chapter 6, the dodecylamine-germinated spores did not become fully phase-dark, suggesting that dodecylamine treatment does not lead to completion of the germination process.

Table 7.2: Spore germination of NCTC 11219 strains for 4 h at 30°C in different germinant mixtures that have been previously described for gIICb. Germination was determined by plate counting after the germinated spores were inactivated by a heat treatment (65°C/10 min). Data for L-alanine/L-lactate/$NaHCO_3$ are from Fig. 7.1. Because spores became heat sensitive in carbonate buffer at pH 9.0, the effect of germinants in this buffer was observed with phase-contrast microscopy. (ND: not determined)

Nutrient mixture in 100 mM Tris-HCl buffer pH 7.4	**Log germination (log CFU/ml)**		**Reference**
	Δ*bont* Δ*pyr*	**Δ*bont* Δ*pyr* Δ*gerBAC***	
L-alanine + L-lactate + $NaHCO_3$ (all 50 mM)	1.3 +/- 0.3	1.5 +/- 0.6	[122]
L-serine + L-lactate + $NaHCO_3$ (all 50 mM)	2.0 +/- 0.0	2.0 +/- 0.2	[122]
L-cysteine + L-lactate + $NaHCO_3$ (all 50 mM)	1.0 +/- 0.1	1.4 +/- 0.5	[122]
L-threonine (100 mM) + L-lactate (50 mM) + $NaHCO_3$ (50 mM)	1.3 +/- 0.6	1.0 +/- 0.5	[122]
Inosine (20 mM) + L-alanine (25 mM) + $NaHCO_3$ (60 mM)	1.3 +/- 0.4	1.3 +/- 0.4	[122,123]
Glucose (20 mM) + L-alanine (25 mM) + $NaHCO_3$ (60 mM)	0.1 +/- 0.2	ND	[123]
Glycine (50 mM)	0.1 +/- 0.2	ND	[123]
Glycine + L-lactate + $NaHCO_3$ (all 50 mM)	0.1 +/- 0.3	ND	[122]
Nutrient mixture in 100 mM carbonate buffer pH 9.0	**Phase-dark spores after 4 h**		
	Δ*bont* Δ*pyr*	**Δ*bont* Δ*pyr* Δ*gerBAC***	
L-alanine (100 mM) + $NaHCO_3$ (50 mM)	< 5%	ND	[123]
L-cysteine (100 mM) + $NaHCO_3$ (50 mM)	< 5%	ND	[123]
L-serine (100 mM) + $NaHCO_3$ (50 mM)	< 5%	ND	[123]
L-threonine (100 mM) + $NaHCO_3$ (50 mM)	< 5%	ND	[123]
Non-nutrient mixture in 100 mM Tris-HCl buffer pH 7.4	**Log germination (log CFU/ml)**		
	Δ*bont* Δ*pyr*	**Δ*bont* Δ*pyr* Δ*gerBAC***	
Dodecylamine (3 mM)	3.3 +/- 0.6	3.9 +/- 1.1	[131,144]

7.3.5 Functional analysis

We assembled previously published reads of 152 gIICb strains. Out of the 152 strains, 17 strains were removed from the analysis due to poor assembly performance (N50 < 10,000). The functional analysis of the 135 remaining strains revealed that across all the strains the *gerBAC* genes are strictly conserved with intact start and stop codons.

7.4 Discussion

This work is the first to report on the role of germinant receptors in group II *C. botulinum*. Analysis of WGS of 24 gIICb strains (18 of toxin type E, 4 of toxin type B, and 2 of toxin type F) previously indicated the presence of a single locus of the GerX3b type in all these strains [120]. The classical concept of spore germination states that germination is triggered by the specific binding of a germinant molecule to a cognate GR protein in the spore membrane. However, it is difficult to understand how the single GerX3b receptor in gIICb strains could have specific binding sites for the large variety of nutrients that can trigger spore germination in this organism. As a first step to unravel the precise role of the GerX3b receptor in germination, we therefore undertook to delete the entire *gerBAC* locus encoding the three receptor subunits, using a gene replacement technique that we used previously to delete the *bont* gene (Chapter 4).

The entire *gerBAC* locus was successfully deleted and replaced by a spectinomycin resistance marker, as confirmed by sequencing of specific PCR amplicons of the region and WGS analysis. Much to our surprise, the deletion did not affect germination induction by any of four major nutrient germinant mixtures, nor by the non-nutrient dodecylamine. Specifically for L-alanine, we demonstrated that the germination response is stereospecific, since D-alanine (in combination with L-lactate) did not induce germination. This is in line with the notion that germinants in gIICb, as in other spore-formers, induce spore germination by interaction with a specific receptor. However, our results clearly demonstrate that GerBAC is not a functional GR, and thus lead to the conclusion that one or more other, so far unidentified, GRs must be responsible for nutrient induced germination.

One possible alternative receptor are the CspC orthologues in gIICb, since a non catalytically active CspC variant was demonstrated to act as GR in *C. difficile* [126]. *cspC* and *cspBA* are located directly upstream of *sleC* in *C. difficile*, and the gene upstream of *sleC* in *C. botulinum* NCTC 11219 also encodes a predicted subtilase family protein, although the similarity to CspC from *C. difficile* is low (32 % amino acid identity over 76 % of the sequence). In addition, this putative protease is predicted to contain an intact catalytic triad Asp/Ser/His, as opposed to CspC of *C. difficile* in which two of the three catalytic residues are lost. Additionally, BLAST analysis revealed five other gene products in *C. botulinum* NCTC 11219 showing low but significant similarity to CspC of *C. difficile* R20291. In the genomes of gIICb strains type E Beluga, type E Alaska E43 and type B Eklund 17B, the number of CspC orthologues is 5, 6 and 10,

respectively. All these predicted proteins are annotated as members of the subtilase family, and contain an intact catalytic triad.

Since our results suggest that GerX3b is not a functional GR, and in the assumption that this is the case in all gIICb strains, one would expect the *gerBAC* genes to have accumulated loss of function mutations in some strains. In complement to the 24 WGS analysed by Brunt et al. (2016) that appear to have intact *gerBAC* genes [245], we extended the search space with 135 gIICb strains and showed that all had intact *gerBAC* genes. Thus, it appears that maintenance of an intact *gerBAC* locus is important in gIICb. We are not aware that alternative functions have been reported for Ger-type receptors in any spore-forming bacteria, and it will therefore be interesting to explore such functions in gIICb. On the other hand, the quest for the genuine GR in gIICb is open. As discussed above, the CspC-like proteins are possible candidates, and their large number in gIICb is compatible with the large variety of germinants in gIICb. However, if they show functional redundancy and hierarchy, as is the case for the multiple Ger receptors in *B. cereus*, their functional analysis will be a difficult task. On the other hand, the possibility of an entirely novel class of GRs should also be considered, and it seems useful therefore to isolate and analyse non-germinating mutants to investigate this possibility.

Chapter 8

SleB is not essential for cortex hydrolysis during germination of *C. botulinum* NCTC 11219 spores

8.1 Introduction

Cortex hydrolysis is an essential step in the completion of spore germination because only when the spore cortex PG has been cleaved, the core can fully rehydrate whereafter vegetative metabolism can be resumed (see 2.2.1.2). Most *Bacillus* species have two CLEs (CwlJ and SleB) of which either one is sufficient for completion of germination. In contrast, most clostridia contain SleB and SleC, and in some species SleM and CwlJ can be additionally present [127]. Brunt et al. (2016) analysed 24 genomes of gIICb strains and found, next to *sleB* and *sleC*, additional *sleC*-like genes designated as *sleC*2a/b (with *sleC2b* mostly present in type E strains), whereas *cwlJ* appears to be absent [120]. However, the functionality of SleB has been questioned, because gIICb strains do not appear to encode a YpeB protein, which in *Bacillus* is required for proper localization and/or function of SleB [139].

Up to now, only a few studies have been performed on clostridia that investigated the functionality of these CLEs. Mutational studies showed that SleC is the essential cortex hydrolase in both *C. perfringens* and *C. difficile*, which additionally possess SleM and a SleB homolog respectively [140,141]. Here, we have attempted to study the functionality of SleB and SleC in strain NCTC 11219 by knockout mutagenesis. The same strategy was used that has been described for deletion of *bont/E* (Chapter 4 and 5) and deletion of *gerBAC* (Chapter 7).

8.2 Materials and Methods

8.2.1 Bacterial strains, growth conditions and spore production

For construction of the *sleB* and *sleC* mutants, we used the atoxigenic and uracil auxotrophic mutant *C. botulinum* NCTC 11219 Δ*bont*::*ermB* Δ*pyrE* (in short referred to as NCTC 11219 Δ*bont* Δ*pyr*), of which the construction has been described in Chapter 4. In addition, *C. botulinum* NCTC 8266 Δ*bont*::*ermB* Δ*pyrE* (in short referred to as NCTC 8266 Δ*bont* Δ*pyr*) was also used for creation of a *sleC* mutant. Growth media used for clostridial and *E. coli* cultures as well as the preparation of spore crops was performed are described in 4.2.1 and 4.2.2.

8.2.2 Plasmid construction

The plasmids pMTL84151Δ*sleB* and pMTL84151Δ*sleC* were constructed to respectively replace the *sleB* and *sleC* genes with the Sp resistance marker *aad9* in strain 11219 Δ*bont* Δ*pyr*. Both plasmids are based on pMTL84151Δ*bont* (shown in Fig. 4.1), which was linearized (i) with PCR using primers P_openF/P_openR for the *sleB* construct, and (ii) with restriction enzymes KpnI and XhoI for the *sleC* construct. The linearized plasmid backbone carries the *catP* marker that encodes Cm/Tm resistance as well as p_{fdx} *pyrE*, that can be used as a negative selection marker in the presence of 5-FOA. Further, in this backbone ~ 1 kb up- and downstream flanking loci of the target gene (*sleB* or *sleC*) were cloned with the *aad9* marker in between using Gibson Assembly. Table 8.1 shows all primers used for plasmid construction and for confirmation of mutants. Primers pairs sleB5'F and sleB5'R, aad9_F/SleB and aad9_R/SleB, sleB3'F and sleB3'R were used for construction of pMTL84151Δ*sleB*, and primer pairs sleC5'F and sleC5'R, aad9_F/SleC and aad9_R/SleC, sleC3'F and sleC3'R for construction of pMTL84151Δ*sleC*.

The assembled Gibson products were then transferred to *E. coli* NEB #C2987 (New England Biolabs) by chemical transformation (following the Gibson protocol), and transformants were selected on LB supplemented with Sp and Cm. After verification of the constructs pMTL84151Δ*sleB* and pMTL84151Δ*sleC* by PCR and sequence analysis, the plasmids were transferred to *E. coli* CA434 by electroporation, which was then used as conjugation donor to transfer the plasmids to *C. botulinum* NCTC 11219 Δ*bont* Δ*pyr*.

8.2.3 Conjugation and isolation of Δ*sleB* and Δ*sleC* deletion mutants

Conjugation of plasmids was performed as described previously (see 4.2.4). After conjugation of pMTL84151Δ*sleB* or pMTL84151Δ*sleC* to strain NCTC 11219 Δ*bont* Δ*pyr*, transconjugants were selected on RCM supplemented with Tm and Cy. Purified transconjugants were then tested for Sp resistance and 5-FOA sensitivity (verifying expression of the *aad9* resistance marker and *pyrE* respectively).

Subsequently, transconjugants were grown and subcultured daily in TPGY supplemented with Sp, and 200 µl of each serial culture was plated on TPGY with Sp and 5-FOA, to select for clones in which double homologous recombination with the flanking loci of the target gene (*sleB* or *sleC*) as well as loss of the plasmid had occurred. Plasmid loss was subsequently confirmed by verifying Tm sensitivity.

Tabel 8.1: PCR oligonucleotides used for cloning and construct verification. The small letters indicate the overhang region of the primer, necessary for annealing fragments via Gibson assembly.

Name	Sequence (5'-3')
ΔsleB	
P_openF	CCGTCGTTTTACAACGTC
P_openR	CTCCTACTTAGCACCATATTC
sleB5'F	GCCCAAAAAGAGCCTTATATACATAAATTAA
sleB5'R	gttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggATTTGATGAAAATGATGCCAAACTCG
aad9_F/SleB	gtctaataaatataattagattggtcattatttttattaagatttAATAAAACAAAAAAATTGAAAAAAGTGTTTC
aad9_R/SleB	catataaatattaatttatgtatataaggctcttttgggcCAATGAATAGGTTTACACTTACTTTAGTTT
sleB3'F	ctagaatagatgaatattacaaagaatatggtgctaagtaggagGACATAATGCTTATACAAGAAATAATAGG
sleB3'R	AAATCTTAATAAAAATAATGACCAATCTAATTA
upSleB	TCATATTGTTACACAGGGTCTTATGCTG
downSleB	TGGTTATGATAAATTTTTCAACAG
sleBinternF	ACAATGTTGCTTATGCTCAAGTATTAGGTGAC
sleBinternR	TGCCGTGCTAGGATTATAAAAGAATAATGC
ΔsleC	
sleC5'F	gatgaatattacaaagaatatggtgctaagtaggagctcggtaccTGAAGTTAAAATAGGTAAAAATCAG
sleC5'R	CACTAACTCCTTAATATTTTTCCTACG
aad9_F/SleC	cttatctcatataatattcgtaggaaaaatattaaggagttagtgCAATGAATAGGTTTACACTTACTTTAGTTT
aad9_R/SleC	ggaatataaaaaagcaatgcttcaaacaatttgaagcattgcTTCTAAAATCTGATTACCAATTAGAATG
sleC3'F	GCAATGCTTCAAATTGTTTGAAGC
sleC3'R	aacgacggccagtgccaagcttgcatgtctgcaggcctcgagGTCAGGAATTCAAAGGAATGG
upSleC	AGTGGGTACTGGAAATCAAGGTG
downSleC	TCATCTGGAAAAGGTTCTGG
SleCintern_F	GGTTCCATATAGTGTTTATG
SleCintern_R	TGCATTACTAGACCATGTAG

8.2.4 Germination assays

Germination was induced by the gerMix L-alanine/L-lactate/$NaHCO_3$ (all at 50 mM) in 100 mM Tris-HCl (pH 7.4) and apparent germination by the non-nutrient dodecylamine (3 mM) in the same buffer. After 4 h incubation of the spores with the germinants at 30 °C, germination was assessed based on loss of heat resistance and DPA release, as respectively described in 7.2.6 and 7.2.7. Strain differences in the extent of germination and DPA release were statistically analysed using the Student's T-test with a significance level of 0.05. Microscopy pictures were assessed as indicated in 7.2.8.

8.3 Results and Discussion

8.3.1 Construction of the Δ*sleB* deletion mutant

The knockout strategy used was already described in detail for deletion of *bont/E* and deletion of *gerBAC* (Chapters 4 and 7). After introduction of plasmid pMTL84151Δ*sleB* into strain NCTC 11219 Δ*bont* Δ*pyr*, three rounds of propagation in TPGY broth with Sp were required before 5-FOA/Sp resistant clones could be isolated of which one clone was further confirmed to be a Δ*bont* Δ*pyr* Δ*sleB* mutant (in short: Δ*sleB*). Plasmid loss was confirmed by the Tm sensitivity of the clone, whereas PCR and sequence analysis with primers up_sleB/down_sleB, annealing outside the homologous loci used in recombination, confirmed the replacement of *sleB* by *aad9* in the genome. In addition, a PCR on gDNA of the Δ*sleB* mutant with primers sleBinternF/ sleBinternR located internally of the *sleB* gene resulted in no PCR product, while a PCR product could be formed on Δ*bont* Δ*pyr* gDNA. This additionally confirms absence of *sleB* in the Δ*bont* Δ*pyr* Δ*sleB* genome.

8.3.2 Qualitative evaluation of spore germination of Δ*sleB* spores

Spore viability. Freshly harvested spores were plated on TPGY agar after being heat activated (65 °C/10 min). No significant differences in counts were found between the Δ*bont* Δ*pyr* parent and the Δ*bont* Δ*pyr* Δ*sleB* mutant ($P > 0.05$), with the average spore yield being 7.6 +/- 0.3 log cfu/ml and 8.0 +/- 0.4 log cfu/ml respectively (n=6). The counts of the Δ*sleB* spore suspensions indicate that sporulation is not affected and that the spores are viable and can complete germination on rich medium.

Core rehydration. Microscopy of spores on agar pads was used to observe core rehydration during germination, which can only be completed if the cortex has been degraded. Heat activated Δ*sleB* spores turned phase-dark within 1 h in the presence of nutrient germinants (L-alanine/L-lactate/$NaHCO_3$), but remained phase-bright without germinants (Fig. 8.1). Moreover, the rehydrated Δ*sleB* spores were swollen in a similar way as the Δ*bont* Δ*pyr* spores, indicating that the cortex had been degraded. In addition, visual (not quantitative) analysis of time-lapse movies showed no difference in the rate of spore germination between the parental and Δ*sleB* strain.

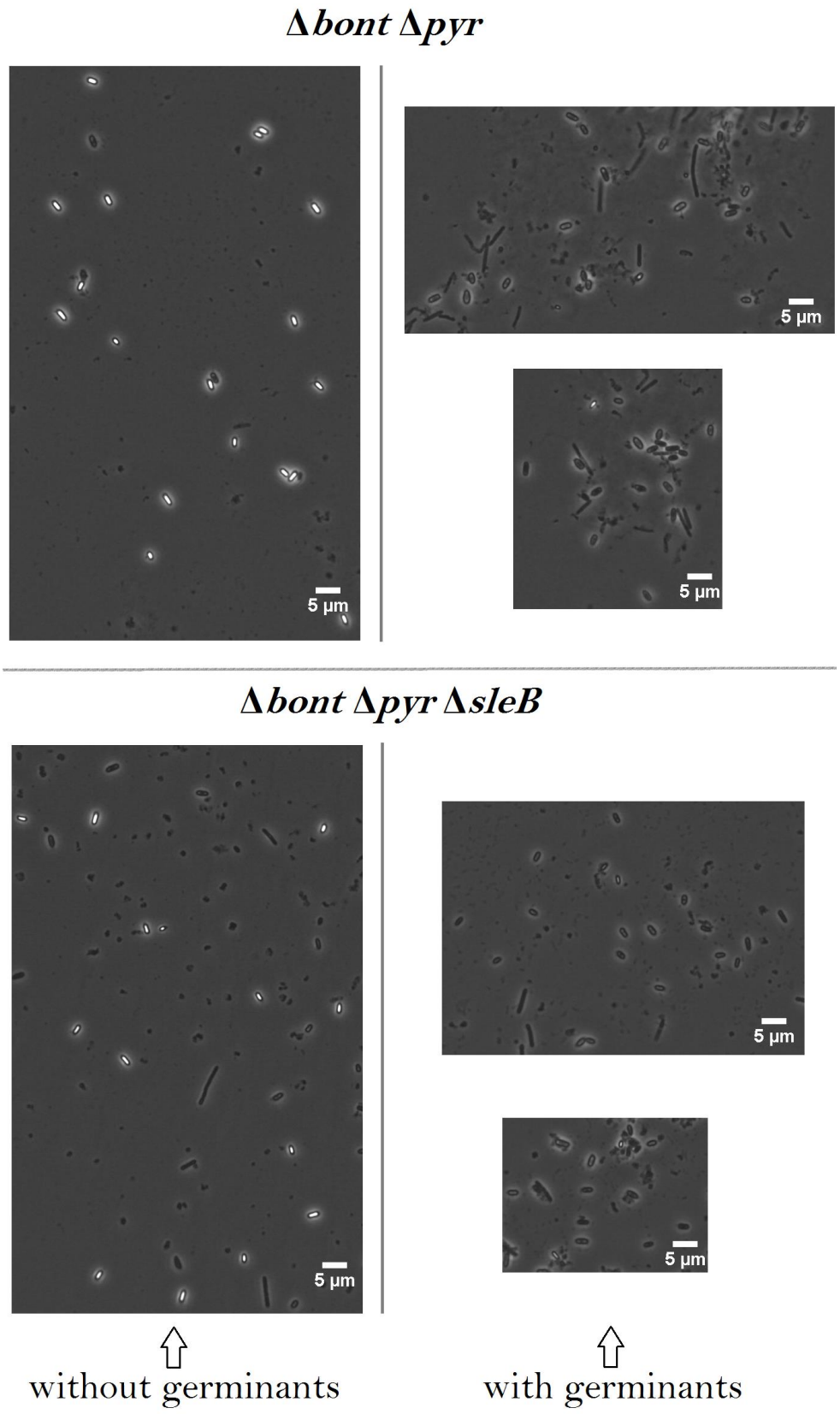

Fig. 8.1: Microscopy images of spores of the parental strain, NCTC 11219 Δ*bont* Δ*pyr* and the mutant strain, NCTC 11219 Δ*bont* Δ*pyr* Δ*sleB* on agar pads without and with germinants (L-alanine/L-lactate/$NaHCO_3$, all at 50 mM). Pictures were taken 1 h after the spores were placed on the pads. Six microscopic fields were assessed for each (with around 200 spores per field), and a representative image is shown.

The high spore viability on agar plates and the core rehydration shown by microscopy, indicate that SleB is not an essential CLE for completion of cortex hydrolysis in *C. botulinum* NCTC 11219 spores. Possibly SleB plays an auxiliary role in germination, but this should be investigated further by the use of kinetic studies and by construction of mutants in which multiple CLEs are inactivated together.

We then investigated whether other germination events are affected due to the *sleB* deletion.

8.3.3 Quantitative evaluation of nutrient and dodecylamine induced germination of Δ*sleB* spores

The loss of heat resistance and the release of DPA were assessed in the presence of the gerMix and dodecylamine (Fig. 8.2). Germination in the nutrient mixture was reduced ($p < 0.05$) for the Δ*sleB* spores (0.3 +/- 0.2 log cfu/ml) in comparison with the parental spores (1.4 +/- 0.5 log cfu/ml), alhough the DPA release was not significantly different between both strains (Δ*sleB*: 53.2 +/- 2.0 % ; parent: 63.1 +/- 10.8 %). Furthermore, the apparent germination induced by dodecylamine was also significantly different between both strains, with 4.6 +/- 0.4 log cfu/ml for Δ*bont* Δ*pyr* spores and 2.6 +/- 0.7 log cfu/ml for Δ*sleB* spores ($p < 0.05$). Surprisingly, the DPA release showed an opposite trend, with significantly more release for the Δ*sleB* spores (90.3 +/- 9.4 %) than for the parental spores (58.8 +/- 5.8 %) ($p < 0.05$). This DPA release is measured after incubation for 4 h at 30 °C in the presence of dodecylamine, but before the spores have received the 65 °C-treatment to inactivate the germinated fraction. When DPA release was measured after heat treatment, the difference between both strains disappeared (Δ*sleB*: 96.0 +/- 2.2 % ; parent: 91.5 +/- 2.3 %).

The observed differences between nutrient induced germination of Δ*sleB* spores and the parental spores, were rather unexpected because the same Δ*sleB* spores showed to rehydrate and swell rapidly on agar pads containing the nutrients (Fig. 8.1). It is unclear why the Δ*sleB* spores in the germination assay, i.e. when incubated in a liquid buffer with germinants, did not become heat sensitive, nor phase-dark (observed with microscopy, picture not shown). However, it should be noted that we have been confronted with variable germination efficiencies before, even for NCTC 11219 Δ*bont* spores, and in our experience germination efficiency can vary significantly between different spore batches and also over storage time, with freshly harvested spores often showing only limited germination. In spore batches showing poor germination immediately after harvest, the germination degree on agar pads always tends to be higher than in liquid, for unknown reasons.

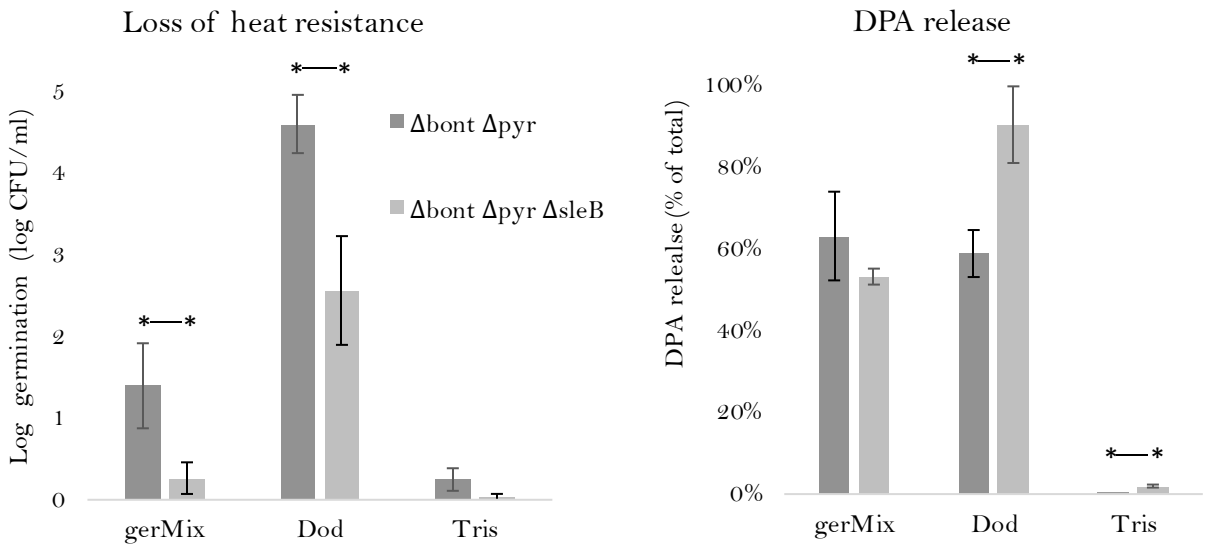

Fig. 8.2: Spore germination of the parental strain, NCTC 11219 Δ*bont* Δ*pyr* and the mutant strain, NCTC 11219 Δ*bont* Δ*pyr* Δ*sleB*. After incubation of heat activated spores for 4 h at 30 °C in the presence of gerMix (L-alanine/L-lactate/$NaHCO_3$), dodecylamine and Tris-HCl buffer, germination was assessed based on loss of heat resistance (Left) and by DPA release (Right). Mean percentages +/- standard deviations are shown of three experiments using independent spore crops. Significant differences ($P < 0.05$) between the two strains are indicated with *.

For dodecylamine induced apparent germination, it is difficult to conclude on the role of SleB, because opposite trends were observed for the loss of heat resistance and DPA release. Dodecylamine germination is believed to have an effect on the SpoVA proteins [131] which mediate DPA release, but an interaction with SleB has not yet been reported. To confirm the involvement of SleB in dodecylamine germination, the genetic complementation of the Δ*sleB* spores will be helpful. Futhermore, a more detailed study on the location of SleB in the spore membranes, could presumably give more insight.

8.3.5 Attempts to construct a Δ*sleC* deletion mutant

Although the same knockout strategy was used as for the deletion of *bont/E*, *gerBAC* and *sleB*, we were not able to isolate a *sleC* deletion mutant. The plasmid pMTL84151Δ*sleC* (Sp^RTm^R), created for the deletion of *sleC*, was successfully conjugated to strain NCTC 11219 Δ*bont* Δ*pyr* and the transconjugants became 5-FOA sensitive as well as Sp/Tm resistant. When these transconjugants were propagated in broth (with Sp, without 5-FOA) and subsequently plated on TPGY with Sp and 5-FOA, about a dozen of colonies appeared. However, these colonies were still Tm resistant, indicating that they had not lost the plasmid (containing *pyrE*) despite being 5-FOA resistant. A total of around 200 clones were picked up from different independent experiments, but all were 5-FOA/Sp/Tm resistant. The presence of the plasmid was additionally confirmed in some of these clones by PCR using vector primers mcsF/mcsR (Table 4.1).

After many failed attempts to obtain a NCTC 11219 Δ*bont* Δ*pyr* Δ*sleC* mutant, the same strategy was employed in NCTC 8266 Δ*bont* Δ*pyr*. The pMTL84151Δ*sleC* plasmid was transferred to this strain, and hereafter, six rounds of serial propagation of the transconjugant were required before 5-FOA/Sp resistant clones appeared (while in every round, 200 µl of culture was plated). Unfortunately, again these clones showed to be Tm resistant and thus they were false positives.

To determine the cause of the 5-FOA resistant phenotype, the *pyrE* (plasmid-based) genes of four 5-FOA/Sp/Tm resistant clones derived from the NCTC 11219 transconjugants and six of the NCTC 8266 transconjugants were amplified with PCR (primers mcs_F and checkC_R) and sequenced. Surprisingly, no mutations were found in the *pyrE* genes of these transconjugants. Therefore, the *pyrF* gene (genome-based) was analysed in the same clones (primers pyrF_F/pyrF_R) because besides PyrE, also PyrF (an orotidine 5-phosphate decarboxylase) plays a role in converting the uracil analogue 5-FOA into a toxic metabolite, and thus cells with an inactive PyrF also become 5-FOA resistant. All sequenced clones indeed showed mutations in *pyrF* (Table 8.2).

Table 8.2: Mutational changes in 5-FOA resistant transconjugants containing pMTL84151Δ*sleC*. Four clones were sequenced from strain NCTC 11219 and six clones from NCTC 8266. (fs: frameshift)

Transconjugant	**Mutation**	**Effect on PyrF**	**Number**
NCTC 11219 Δ*bont* Δ*pyr*	C→A	Ala59Glu	3
	TAAAAAA deletion	Asn36fs	1
NCTC 8266 Δ*bont* Δ*pyr*	TAAAAAA deletion	Asn36fs	2
	T insertion	Tyr80fs	1
	G→C	Cys20Ser	1
	TG deletion	Asp57fs	2

The reason why deletion of *sleC* did not work is not clear. One possible explanation is that the frequency of recombination with the homologous arms flanking the *sleC* gene was lower than it was for the homologous arms flanking the *sleB*, *gerBAC* and *bont/E* genes. Although the length of the homologous arms was similar in all cases, the recombination efficiency may also depend on the sequence. An alternative explanation is that *sleC* is an essential gene for vegetative cell growth. This might be the case if SleC would be involved in peptidoglycan metabolism of the vegetative cells rather than in cortex hydrolysis. One way to test this could be to produce SleC recombinantly, and test the activity of the purified enzyme on decoated spores and on vegetative cells.

8.4 Conclusion

In this chapter we investigated the functionality of two CLEs in gIICb, SleB and SleC, in spore germination. First, a NCTC 11219 Δ*bont* Δ*pyr* Δ*sleB::aad9* mutant was constructed. The spore yield in this mutant was not affected, and the plating efficiency of the spores was a first indication that they could still complete germination and grow out on rich medium without SleB. Microscopy additionally indicated that Δ*sleB* spores rapidly turn phase-dark in the presence of the germinant mixture L-alanine/L-lactate/$NaHCO_3$, while they remained phase-bright without these nutrients. No differences were found between Δ*sleB* spores and the parental spores, suggesting that SleB is not essential for cortex hydrolysis. This is similar to *C. difficile*, in which the inactivation of *sleB* had no effect on spore germination [141]. However, our microscopy images have not been quantitatively nor kinetically analysed so an auxiliary role of SleB can not be excluded.

Next, we investigated whether other germination-associated events are affected due to the *sleB* deletion. Germination in the liquid nutrient mixture was significantly reduced for the mutant spores, although DPA release was not different in comparison with the parental spores. In addition, dodecylamine apparent germination was affected, but because the loss of heat resistance and DPA release were apparently contradictory, the results were not conclusive. Therefore, these experiments should be repeated in the future, before it can be concluded that SleB plays a significant role in the cascade of germination events. Furthermore, complementation of the Δ*sleB* spores will aid to clarify SleB's functionality.

Unfortunately, the inactivation of SleC could not be accomplished, because false positive 5-FOA resistant clones, of which was demonstrated that they had mutations in *pyrF*, hindered the isolation of the correct *sleC* mutant. Although the same knockout strategy was used as for *bont*, *gerBAC* and *sleB*, it is unclear why the recombination efficiency was too low in this case.

Chapter 9

General conclusions and future perspectives

THE ILLNESS BOTULISM IS A SIGNIFICANT PUBLIC HEALTH HAZARD because the botulinum neurotoxin (BoNT) that is responsible for the disease is the most potent biological substance known to mankind. BoNT causes flaccid paralysis, eventually resulting in death when untreated. Because of its extreme potency, the toxin is even considered a potential bioterrorism weapon. Therefore, most attention in research has been given to the disease and the toxins, while the organism *C. botulinum* that produces BoNT, has been studied less intensively. Nevertheless, because *C. botulinum* poses a tremendous concern for the food industry, food processing and preservation methods have been well established to control group I and II *C. botulinum* (which are mainly associated with human botulism) in foods. While group I strains are the main target in canning of low acid foods because of their high spore heat resistance, group II strains have a lower spore heat resistance but they can grow at temperatures as low as 3.3 °C, making them the pathogen of concern in REPFEDS. In this PhD thesis we focused on gIICb strains, and more specifically, on the mechanism of spore germination in these strains.

To avoid the stringent biosafety procedures that apply to working with *C. botulinum*, we aimed to construct nontoxigenic mutants. Although targeted gene inactivation in clostridial species has proven to be a rather difficult and inefficient task for a long time, the genetic toolbox for knockout mutagenesis has expanded the last few years. Especially the ClosTron insertion mutagenesis system has been applied since 2007 in many clostridia, including one gIICb strain (Eklund 17B), in which *bont* was insertionally inactivated. We tried to obtain this nontoxic mutant, but according to the US FDA, this strain cannot be regarded as safe because the ClosTron insertion could be lost (though this is highly unlikely) and the *bont* gene be reversed to its active state. Therefore export of this strain from the US was prohibited. We thus obtained toxic strains, *C. botulinum* NCTC 11219, NCTC 8266 and NCTC 8550, all producing BoNT type E, and attempted to construct our own *bont/E* deletion mutants.

FIRST, THE WGS OF THESE THREE STRAINS WERE ASSEMBLED. At the start of this PhD thesis, only two WGS were available from type E strains: *C. botulinum* E1 strain Beluga (six contigs) and strain Alaska E43 (whole genome as one contig). We have demonstrated in Chapter 3 that NCTC 8266 and NCTC 8550 are highly similar to strain Alaska (99 % identity), whereas NCTC 11219 differs more (93 % identity). For that reason, we were unable to assemble the latter as a whole genome, but instead four contigs were accomplished, applying a combinatorial approach of reference assembly against Alaska E43, *de novo* assembly of non-assembled reads and manual editing. The very close similarity between NCTC 8550, NCTC 8266 and Alaska is remarkable, given the fact that they have been isolated

in different years and geographical locations (NCTC 8266: Nanaimo, British Colombia (1944); NCTC 8550: Institute Pasteur, France (1952); strain Alaska E43: Alaska (year unknown)). It raises the question whether accidental strain switching or misnaming may have occurred.

Construction of deletion mutants in *C. botulinum* had not yet been accomplished at the start of this research, and therefore we took up this challenge and **DEVELOPED A NEW KNOCKOUT STRATEGY**, with which we successfully constructed NCTC 11219 Δ*bontE::ermB*, in addition to a ClosTron mutant, NCTC 11219 *bontE*211a::CT (Chapter 4). Both mutants were characterized to assure that all phenotypical properties were unaffected, except for toxin production. While most characteristics of the mutants showed to be identical to the parental strain, also some differences were noted. Heat resistance of the spores at 70 °C was slightly lower for both mutants, and growth of the deletion mutant in 2.3 % NaCl was faster than for the other strains. In addition, an early onset of sporulation was demonstrated for the ClosTron mutant. However, the differences were small and the properties of the mutant strains were still well within the normal range for gIICb strains. Therefore, we considered the NCTC 11219 Δ*bontE::ermB* mutant to be a valid safe surrogate to facilitate our research and we used this strain to study germination in Chapters 6 to 8.

Nevertheless, in Chapter 5, we additionally investigated whether one or more of the altered properties of the NCTC 11219 mutants were related with the inactivation of BoNT production, or due to spontaneous mutations that had occurred during mutant construction. Therefore we constructed a *bont/E* insertion and deletion mutant in another strain, NCTC 8266, in an identical way as decribed for NCTC 11219, and characterized their properties in comparison with the wild type strain. Here, no phenotypical differences were observed for the NCTC 8266 deletion mutant, whereas a growth defect at 8 °C and 12 °C was shown for the ClosTron mutant. Moreover, **WGS ANALYSIS WAS PERFORMED** on the Δ*bontE::ermB* and *bontE*211a::CT mutants of strain NCTC 11219, as well as on NCTC 8266 Δ*bontE::ermB*. Unexpectedly, the analysis showed a lot of mutations including three large deletions (18 kb, 51 kb, 20 kb) in the NCTC 11219 deletion mutant, while this was not the case for the NCTC 8266 deletion mutant, in which only three mutations were found (besides one mutation that was introduced on purpose). The higher mutational rate in NCTC 11219 in comparison to NCTC 8266, could possibly be related with the higher amount of prophage regions (Chapter 3). Such regions are known to be less stable and it is exactly in these regions that the large deletions occurred. Because the gene replacement strategy comprises many different steps (deletion of *pyrE*, double recombination with flanking loci of *bont/E*, restoration of *pyrE*), the probability of adventitious mutations to occur is relatively high, and it is recommended to

conduct WGS analysis to document these mutations and verify that no important gene functions have been affected. In addition to the NCTC 11219 deletion mutant, we concluded that also the NCTC 8266 deletion mutant is very useful for challenge testing. However, it should always be kept in mind that both mutants might react differently from the wild type in not tested situations. These strains will make challenge testing more accessible for food producing companies, and several companies have already expressed an interest. However, more nontoxigenic strains should be constructed, because challenge testing optimally requires use of a strain cocktail of gIICb type B, E and F strains to account for sufficient strain-to-strain variability.

The early onset of sporulation of NCTC 11219 *bontE*211a::CT could possibly be linked to a mutated diguanylate cyclase, since this was the only mutation found in this strain. These enzymes are involved in the synthesis of the secondary messenger c-di-GMP, which plays a role in the regulation of various lifestyle changes in different bacteria. However, a link with sporulation has only been demonstrated very recently by Fagerlund et al. (2016), who showed that the inactivation of a diguanylate cyclase resulted in a 2.5-fold higher sporulation efficiency after twenty hours of growth in *B. thuringiensis* [202]. In comparison, the sporulation efficiency reported in Chapter 4 for the NCTC 11219 ClosTron mutant was increased about hundredfold compared to the wild type strain after ten hours of growth. This is a remarkable finding, and the role of c-di-GMP in sporulation onset in gIICb should be confirmed and further studied. By directed evolution experiments, we have already attempted to obtain spontaneous mutants of the wild type strain that showed a significant advanced sporulation onset, but unfortunately this was not successful. Further attempts using modified selection regimens should be tested, and if such early sporulation mutants can be isolated that show one or more mutation(s) in diguanylate cyclase encoding genes, this could corroborate our finding that c-di-GMP signaling is involved in sporulation. Furthermore, it would be useful to construct a diguanylate cyclase knockout, for example by using ClosTron mutagenesis. However, because of the large number of GGDEF-domain containing proteins, which are believed to encode for diguanylate cyclases, in gIICb strains (six in both NCTC 11219 and NCTC 8266), their functional analysis could be a difficult task because they may be functionally redundant. Nonetheless, further research on this topic could provide an interesting novel insight in the complex developmental program of bacterial sporulation.

IN CHAPTERS 6 TO 8, WE FOCUSED ON SPORE GERMINATION IN GIICB.

INDUCTION OF GERMINATION by different germinants, and inhibition of germination by some natural preservative compounds was studied in NCTC 11219 Δ*bontE::ermB* spores (Chapter 6). While germination induced by nutrients and by high pressure (HP) had been described previously in a few studies, germination induced by the non-nutrients exogenous Ca^{2+}-DPA and dodecylamine had not yet been reported in gIICb. We showed that Ca^{2+}-DPA (20 – 60 mM) was not effective for germination induction. This was somehow to be expected since it is known that Ca^{2+}-DPA activates the cortex hydrolase CwlJ and gIICb strains do not carry a CwlJ homolog. Nevertheless, Ca^{2+}-DPA germination is not always clearly linked to CwlJ, since *C. difficile* spores do not germinate with Ca^{2+}-DPA although they encode a CwlJ homolog [143], while *C. perfringens* spores germinate in response to Ca^{2+}-DPA despite the absence of a *cwlJ* homolog in the genome. Our result suggests that the CLE enzymes in gIICb are not activated by exogenous Ca^{2+}-DPA.

In addition, HP treatments (200/600 MPa, 15 min, 30 °C) did not induce germination based on loss of heat resistance. Nevertheless, HP treatment at 600 MPa induced a small amount of DPA release, and this may indicate a direct or indirect effect on the SpoVA proteins which are believed to mediate DPA release. Our results confirm an earlier report that HP cannot induce gIICb spore germination efficiently at moderate temperatures [229]. Since HP treatments and exogenous Ca^{2+}-DPA induce germination in bacilli and at least in some clostridia, our results show that the germination mechanism can be significantly different between genera and even between species, and with this we highlight the need for specific studies in gIICb strains.

Also in Chapter 6, we found that the gerMix (L-alanine/L-lactate/$NaHCO_3$) and the surfactant dodecylamine were very effective germinants for NCTC 11219 Δ*bontE::ermB* spores, although the mechanisms of germination differed between the two. In both cases, spores lost their heat resistance and released their DPA, but only the nutrient germinated spores rehydrated and swelled fully. In contrast, spores incubated with dodecylamine remained phase-bright and can therefore not reinitiate metabolism. A similar observation has been reported for other spore-formers. In *B. subtilis* and *C. perfringens*, dodecylamine germinated spores only partly lost their refractility, and in *C. difficile* only minimal cortex lysis was demonstrated upon dodecylamine germination [125,143,144]. Although dodecylamine germination probably does not proceed via the normal germination cascade, it is an interesting phenomenon that can learn us more about how the spore core is maintained in a dehydrated state, and how this state can be breached. Dodecylamine, especially at higher temperatures, is

a highly efficient sporicidal and it is currently applied in the product VIRUSOLVE+ (Virusafe, Mansfield, England).

We further studied **THE INHIBITORY ACTION ON GERMINATION** of four natural antimicrobial compounds. Most studies on the use of natural antimicrobials as food preservatives have focused on inhibition of vegetative bacteria. However, since spore germination always preceeds outgrowth, inhibition of spore germination can also potentially contribute to an increased shelf life and improved safety of foods. In addition, since spore germination is an unique physiological process, its inhibition is likely to rely on different mechanisms than the inhibition of vegetative cell growth, and it can be anticipated that there is not necessarily a correlation between the capacity of a compound to inhibit vegetative growth and spore germination. In this work, we investigated the effect of carvacrol, cinnamaldehyde, carrot seed EO and hop β-acids on gIICb spore germination. For carvacrol, cinnamaldehyde and carrot seed EO, the lowest concentration that had an inhibitory effect on nutrient induced spore germination was respectively 40-, 16- and 100-fold lower than the MIC for vegetative cell growth. The inhibitory action of carrot seed EO is in contrast to the situation in *B. cereus*, where the oil showed no effect while ten other EOs did [225]. Besides our present work, there is only one other study, published almost thirty years ago, reporting on the impact of EOs on spore germination on gIICb. In this work, origanum and cinnamon EO (of which carvacrol and cinnamaldehyde are the active compounds, respectively) had a strong inhibitory effect at 50 ppm but not at 10 ppm [226]. In strain NCTC 11219, we still demonstrated a significant inhibitory effect (< 50 % relative log germination) at respectively 8 ppm and 31 ppm of the pure compounds, carvacrol and cinnamaldehyde. It should be noted that the older study determined germination by counting the fraction of phase dark spores with microscopy, whereas we determined germination by plate counting.

Hop β-acids, in contrast, were unable to inhibit germination even at concentrations equal to the MIC for vegetative growth. It will be interesting to see if higher concentrations, in the range where carvacrol and cinnamaldehyde inhibit germination, will have an effect. Nevertheless, the extraordinary low MIC value (2.5 ppm) on gIICb vegetative cells makes the hop β-acids promising antibacterials for food preservation. According to the manufacturer Hopsteiner, 5-10 ppm are effective as an antibacterial agent against many Gram-positives (*Bacillus*, *Listeria*, *Brochrotrix*). In literature, MIC values of 1 ppm were found for *L. monocytogenes* and 3 ppm for *H. pylori* [246,247].

The low concentrations at which the EO compounds inhibit gIICb spore germination are potentially interesting from an applications point of view, for example in pasteurized foods with a long refrigerated shelf life, where gIICb is the major pathogen of concern. The potential of EO compounds is currently limited because they often cause unacceptable sensory deviations at the concentrations needed to inhibit vegetative bacterial growth in foods.

IN CHAPTER 7, WE INACTIVATED GERX3B, the only GR that has been identified in the genome of gIICb strains. This was achieved by the same knockout strategy that was employed for *bont/E* deletion. The presence of only a single receptor contrasts with the large variety of germinants that trigger germination in gIICb and it seemed unlikely that GerX3b could act as a specific receptor for all these germinants. Surprisingly, deletion of the entire *gerBAC* locus, which was confirmed by PCR and whole genome sequencing, had no effect on nutrient induced germination. Different nutrient mixtures were tested, but no differences in germination were seen in comparison with the parental strain (Fig. 7.1 and Table 7.2). Also dodecylamine apparent germination was unaffected by the *gerBAC* knockout. This is in striking contrast with mutational studies in *C. perfringens*, which contains a *gerK* locus that has a similar bicistronic organization to *gerX3* (in addition to a monocistronic *gerAA* located rather far away from *gerK*). Those studies concluded that GerKC is the main receptor protein involved in nutrient germination. GerK of *C. perfringens* was also involved in dodecylamine induced germination, which in bacilli is independent of Ger receptors [124].

Our work is the first study of Ger receptors in gIICb and the lack of a role of GerX3b in germination clearly requires further investigation. A first question is whether GerX3b has any role at all. The finding that the genes are conserved and intact in all 159 available genome sequences of gIICb strains suggests an important function. So far we have focussed on spore germination of the Δ*gerBAC* mutant, but it may be useful to extend the analysis to see if any other properties are affected. Secondly, the quest for the genuine GR(s) in gIICb is now open. The CspC-like proteins are possible candidates, in analogy with *C. difficile*, and their large number in gIICb strains is compatible with the large variety of germinants. On the other hand, the possibility of an entirely novel class of receptors should also be considered, and therefore it will be useful to isolate and analyse non-germinating mutants. At the moment, the optimization of a protocol for random transposon mutagenesis in gIICb is in progress in our lab.

FINALLY, CHAPTER 8 ZOOMED IN ON THE CLES. The *C. botulinum* type E strains that were used in this study contain a putative *sleC* and *sleB* gene, but also an additional *sleC*-

like gene designated as *sleC*2b and a *sleM*-like gene, although the latter is not annotated as a CLE but as a muramidase [120,127]. In *C. perfringens*, SleM is believed to degrade PG-fragments generated by SleC during germination. In this PhD, we focused on the functionality of SleC and SleB by the use of knockout mutagenesis. A *sleB* deletion mutant was successfully constructed and microscopy images demonstrated that spores lacking SleB were still able to degrade the cortex and complete germination on germinant-containing agar pads. However, opposite results were obtained in the quantitative germination assays in liquid germinant solution, in which the germination of Δ*sleB* spores could not be induced. A clear conclusion could also not be made concerning the role of SleB in apparent germination by dodecylamine, because the loss of heat resistance and the DPA release were in conflict. Therefore, while it is clear that SleB is not essential for germination, the experiments are currently insufficiently reliable and reproducible to completely rule out a minor or more subtle role. Further work should be done to make the germination assays (including the production of spore crops) more reproducible. It will also help to include a genetically complemented Δ*sleB* strain.

Although four deletion mutants could be constructed in this thesis by the same strategy, a Δ*sleC* mutant could not be successfully accomplished because false positive 5-FOA resistant clones (having mutations in *pyrF*) hampered the isolation of the correct mutant. It remains unclear why the frequency of double homologous recombination together with the loss of the deletion plasmid in this case was lower than the frequency of mutations to occur in *pyrF*. However, the problem highlights the importance to improve the strategy or to develop an alternative deletion strategy in the future. To this end, the optimization of the highly efficient genome-editing tool, the CRISPR-Cas sytem (CRISPR-Cas system (clustered regularly interspaced short palindromic repeat–CRISPR-associated protein), is currently ongoing in our lab. The strategy has recently been used in various clostridia (*C. beijerinckii*, *C. pasteurianum*, *C. cellulolyticum* and *C. acetobutylicum* [248–250]) and it has been proposed as a simple and effective solution to overcome several barriers in *Clostridium* mutant construction. In the future, we aim to apply this system in gIICb strains to construct more mutants in germination genes.

TO CONCLUDE, the founding of a completely new research line in our lab was a first significant achievement, since only a few research groups study gIICb strains worldwide because of the difficulty to manipulate this organism. This PhD work has contributed to the development of genetic tools for gIICb, and has also provided some novel insights in spore germination in this group of organisms. By the use of a newly developed knockout strategy, we were the first to delete the neurotoxin gene in gIICb, thus providing nontoxigenic strains

that greatly facilitate fundamental and applied research in this organism. Using the same deletion method, knockout mutants were created of the germination genes *gerBAC* and *sleB*, to gain more insight into their role in spore germination of gIICb. Despite the importance of gIICb strains as the main target pathogen for many refrigerated foods, it is remarkable how little is known about the germination mechanism of these strains. A better understanding of this mechanism is not only important to improve the acccuracy of predictive models that are used to assess food safety from production to consumption, it may also allow the development of novel control strategies, since germination is an essential step in the progression of toxin production. Although *B. subtilis* has served for many years as the model organism to study germination, recent findings in several *Clostridium spp.* have demonstrated significant differences. Our work in gIICb has also led to a remarkable and unprecedented finding, i.e. that the only canonical Ger receptor present in gIICb (GerX3) is not involved in nutrient induced germination, although this had been widely assumed by many authors. Our finding leads to the important conclusion that spore germination in gIICb must rely on other germinant receptors, possibly of an entirely novel type. With this, the conclusion of this work offers an exciting prospect for further work to unravel the germinant receptors and other aspects of the spore germination mechanism in this important pathogen.

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